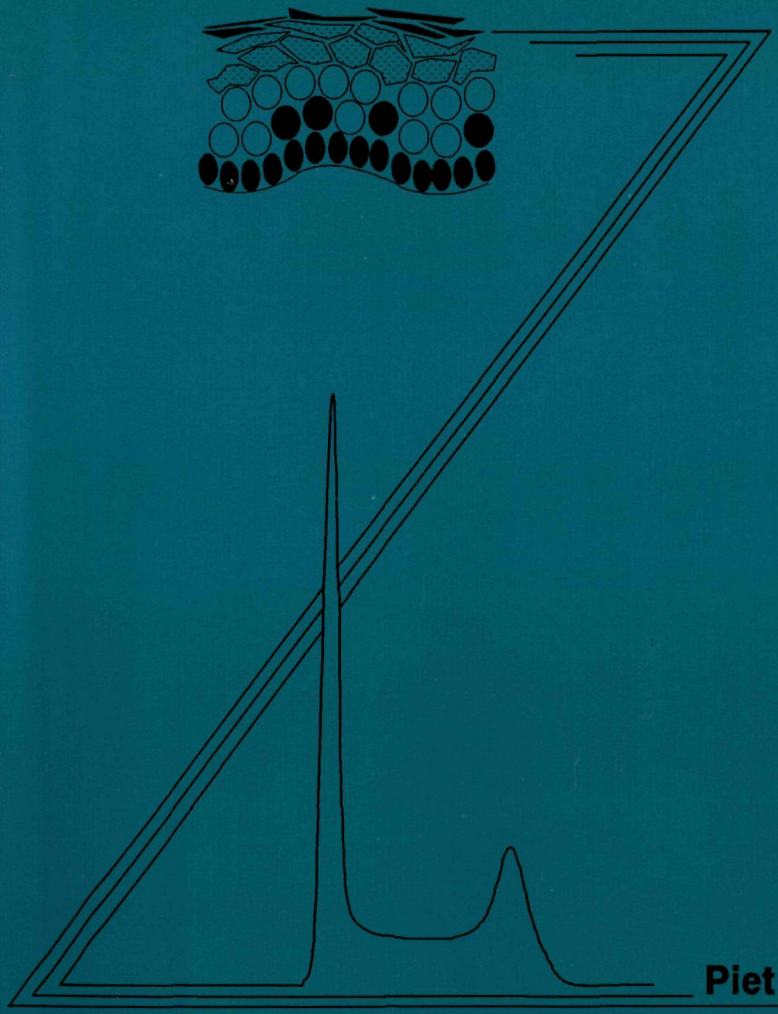
NOVEL CYTOMETRIC TECHNIQUES TO STUDY PROLIFERATION AND DIFFERENTIATION OF HUMAN KERATINOCYTES



Piet EJ van Erp

Drukwerk: Verhaak B.V., Grave

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Erp, Petrus Everardus Josephus van

Novel cytometric techniques to study proliferation and differentiation of human keratinocytes / Petrus Everardus Josephus van Erp. - [S.l. : s.n.]
Proefschrift Nijmegen. - Met lit. opg.
ISBN 90-9004409-4
Trefw.: huid ; cytometrie

NOVEL CYTOMETRIC TECHNIQUES TO STUDY PROLIFERATION AND DIFFERENTIATION OF HUMAN KERATINOCYTES

NOVEL CYTOMETRIC TECHNIQUES TO STUDY PROLIFERATION AND DIFFERENTIATION OF HUMAN KERATINOCYTES

Een wetenschappelijke proeve op het gebied
van de medische wetenschappen,
in het bijzonder de geneeskunde

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit te Nijmegen,
volgens besluit van het college van decanen
in het openbaar te verdedigen
op dinsdag 5 november 1991
des namiddags te 1.30 precies

door

PETRUS EVERARDUS JOSEPHUS VAN ERP
geboren op 24 maart 1956 te Geffen

Promotores: Prof. Dr. P.D. Mier
Prof. Dr. R. Happle (University of Marburg, Germany)

Co-promotores: Dr. J. Schalkwijk
Dr. P.C.M. van de Kerkhof

The research presented in this thesis was performed in the Department of Dermatology, University Hospital Nijmegen, The Netherlands

The publication of this thesis was supported by Stichting Dermatologie Nijmegen, The Netherlands

Aan Ans en Anneke
Ter herinnering
aan mijn vader

Contents

Abbriviations 8

I. GENERAL INTRODUCTION 9

II. INTERMEDIATE FILAMENTS AND DIFFERENTIATION-RELATED PROTEINS AS COMPARTMENT SPECIFIC MARKERS IN THE EPIDERMIS. 29

III. CELL CYCLE KINETICS IN EPIDERMIS USING BrdUrd/IdUrd INCORPORATION 47

IV. EXPRESSION OF THE KI-67 ANTIGEN. ITS RELATION TO THE CYCLING CELL POPULATION IN HUMAN EPIDERMIS 61

V. CHANGES IN INTRACELLULAR pH AS AN EARLY FUNCTIONAL MARKER OF KERATINOCYTE ACTIVATION 81

VI. APPLICATION OF CYTOMETRIC METHODS TO STUDY GROWTH AND DIFFERENTIATION IN NORMAL AND PSORIATIC EPIDERMIS . 95

VII. APPLICATION OF CYTOMETRIC METHODS TO STUDY EPIDERMAL GROWTH AND DIFFERENTIATION IN VITRO 119

VIII. FINAL CONSIDERATIONS 141

IX. SUMMARY,
SAMENVATTING 151

Publications 161

Dankwoord 167

Curriculum vitae 169

ABBREVIATIONS

(list of abbreviations frequently used in this thesis)

ADB	Diacetoxydicyanobenzene
AEC	Aminoethylcarbazole
AM	Acetoxymethylester
BCECF	Bis(carboxyethyl)carboxyfluorescein
BrdUrd	Bromodeoxyuridine
CV	Coefficient of variance
DCH	Dicyanohydroquinone
FCM	Flow cytometry
FITC	Fluorescein isothiocyanate
GAMFITC	FITC-conjugated goat anti-mouse immunoglobulins
GF	Growth fraction
IdUrd	Iododeoxyuridine
LI	Labelling index
MAb	Monoclonal antibody
N_c	Number of cycling cells
N_s	Number of cells in S-phase
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PI	Propidium iodide
PMN	Polymorphonuclear leukocyte
RAMFITC	FITC-conjugated rabbit anti-mouse immunoglobulins
RAMPO	Peroxidase-conjugated rabbit anti-mouse immunoglobulins
RAS	Right angle scatter
RNAse	Ribonuclease
S.D.	Standard deviation
SAMFITC	FITC-conjugated swine anti-mouse immunoglobulins
SNARF-1	Carboxyseminaphthorhodafluor-1
SWARPO	Peroxidase-conjugated swine anti-rabbit immunoglobulins
T_c	Cell cycle time
T_s	Duration of S-phase
UV	Ultra Violet

CHAPTER I. GENERAL INTRODUCTION

CHAPTER I. GENERAL INTRODUCTION

1. Introduction 11
2. Aim and outline of the thesis 12
3. Cell kinetics in epidermis 12
4. Keratinocyte differentiation 13
5. Models to study epidermal growth *in vivo* and *in vitro* 14
6. Methodology and techniques 15
   i) Use of monoclonal antibodies (15)
   ii) Cell cycle kinetics (16)
   iii) The biological application of fluorescent indicators (22)
7. References 25

1. Introduction

Skin is an organ; "it possesses an unusual shape, a variety of appendages and it encloses or supports all other organs, but it is nevertheless a single integrated organ"[1]. Its main function is acting as a barrier between the internal milieu of the organism and the external environment. However, the organ acts only partly as a physical and static barrier by producing a dead layer of protein and lipid material, but is also a controlling and communicating device with a high degree of organisation.

The outer part of the skin is the epidermis and is composed mainly (90-95%) of keratinocytes. It is this part of the skin which produces the outermost protective barrier of cross-linked protein and neutral lipids. Furthermore, it contains specialized cells with specific functions, such as immunoprotection by antigen-presenting Langerhans cells and protection against UV irradiation by melanin-producing melanocytes. Epidermis is a continuously renewing epithelium; cells in the lower layers are dividing and replacing the differentiated and dead corneocytes which scale off from the skin surface, leaving the organic physical barrier for protection. This process of controlled cell production is called epidermopoeisis.

2. Aim and outline of the thesis

Our knowledge of the etiology of skin diseases characterized by abnormal growth is relatively limited. Even more important, still very little is known of how epidermopoeisis is controlled in normal epidermis. There is no cure for skin diseases due to abnormal growth control, such as psoriasis. Mechanisms are complex, additional models for epidermal growth and differentiation, and specific techniques to analyze these processes, are needed. Therefore we aimed firstly, for the development of novel, additional immunohistochemical and flow cytometric techniques to study epidermal growth. These techniques were primarily focused on the mechanism of "recruitment of resting germinative cells" in response to injury, or in skin disease. Secondly, we investigated the usefulness of keratinocytes grown *in vitro* as model to study this process. The culture system could also serve as a model to test and compare the usefulness, specificity and sensitivity of the newly developed techniques. At a later stage a modification of the *in vitro* model was used to study the multistep process of epidermal differentiation.

3. Cell kinetics in epidermis

Figure 1 illustrates the different phases in the life-cycle of all proliferating mammalian cells. Two stages in the progression of a replicating cell can be recognized without great difficulty. Firstly, the period of DNA synthesis, since the DNA in the cell nucleus is replicated only during a limited portion of interphase. This period is denoted as the S-phase (S=synthesis) of the cell cycle. The other distinct stage is, of course, the cell division phase, which is denoted as the M-phase (M=mitotic). Between these two distinct stages, two "gaps" in the cell cycle exist.

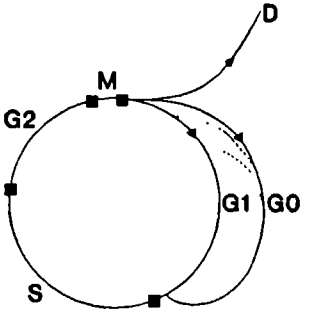


Figure 1 The concept of the cell cycle. When a cell commits itself to cell division it progresses through this cell cycle. Some cells, however, can escape from the cycle and can become quiescent.

The interval between DNA synthesis and the next mitosis is referred to as the G_2-phase (G=gap), and the interval between mitosis and the S-phase is the G_1-phase. G_1-phase and G_2-phase are not less important with respect to function in the cell cycle. During G_2-phase there is biochemical preparation for the incipient mitosis, including assembly of spindle protein, and in G_1-phase biosynthesis takes place of enzymes necessary for DNA synthesis. It is postulated that it is at this point at which cells commit themselves to a further mitosis[2]. Presumably also at this point, cells can enter a resting (G_0) phase or irreversibly commit themselves to terminal differentiation. The total size of the epidermal compartment, and hence total cell production can be regulated by the number of cells cycling, and the rate at which the cells progress through the different cell cycle phases[3].

4. Keratinocyte differentiation

When cells leave the cell cycle and migrate into the upper epidermal layers, they are destined to die. The enucleated (dead) cells finally will form the dead surface layer of the skin (stratum corneum). This process is defined as differentiation. During this process, cells undergo dramatical biochemical and morphological changes (figure 2). Synthesis of structural proteins such as involucrin, and filaggrin and induction of the enzyme transglutaminase are thought to be key steps in this process. Furthermore, basal cell-specific keratins (keratins 5 and 14) are downregulated and new keratins are synthesized (keratins 1 and 10). Together with others, these structural and

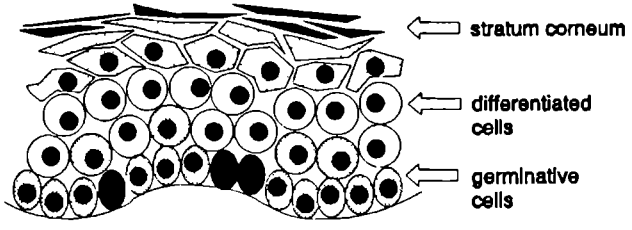


Figure 2 When keratinocytes progress upward in epidermis, they undergo successive morphological and phenotypical changes. Cycling cells (closed circles); Resting cells (shaded circles).

catalytic proteins enable the cells to go through marked morphological and cytostructural changes, including the appearance of various intracellular lamellar bodies and other differentiation-specific structures (membrane coating granules, keratohyalin granules, Odland bodies, etc.), the appearance of desmosomal links, and the occurrence of nucleolytic and proteolytic degradation of the bulk of the intracellular organelles and macromolecules[4].

5. Models to study epidermal growth in vivo and in vitro

Regeneration after tape stripping, and psoriasis which is a common skin disease affecting about 2% of the world population, are well established *in vivo* models for epidermal growth. The repeated application of adhesive tape (tape stripping) in order to remove the stratum corneum is a standard injury of the skin introduced by Pinkus about 40 years ago[5]. About 40 hours after the trauma a (synchronous) cohort of keratinocytes enters the S-phase of the cell cycle and will divide afterwards[6]. This process of hyperproliferation is accompanied by inflammation and abnormal keratinization (parakeratosis). In this respect, the trauma induced by tape stripping has many similarities with psoriasis. Clinically, this skin disease is characterized by three different morphological elements: scaling, thickening, and erythema[7], reflecting abnormal differentiation, hyperproliferation and inflammation.

However, these *in vivo* models have disadvantages. Skin of healthy volunteers and patients is not an unlimited source of material. Especially when developing new techniques, large quantities of homogeneous tissue or cells are needed. Proliferation, keratinization, and inflammation are so interrelated *in vivo* that ascribing specific metabolic pathways, enzyme activities, or protein modifications to one of these processes is extremely difficult. Studies on experimental drugs, mitogens, and carcinogens cannot be undertaken, or must be done *ex vivo*. Maintenance of skin slices, however, is difficult practically, because we do not know what influence the *ex vivo* conditions have on epidermal growth; *ex vivo* conditions are certainly not identical to the *in vivo* situation.

Culturing of human keratinocytes has become a routine procedure in most dermatological research laboratories, using the standard model developed by Rheinwald and Green in 1975[8]. However, this model is not primary developed

for studies of the mechanisms by which growth is regulated, but its main purpose was the production of large numbers of keratinocytes as fast as possible. Nevertheless, in the past decade some elegant studies, especially by Watt and coworkers[9,10,11], have demonstrated the potential value of the model to investigate epidermal growth and differentiation. By decreasing the calcium concentration of the medium or affecting the adherence of the cells to the culture dishes, the differentiation process of keratinocytes can be modulated.

6. Methodology and techniques

The scope of this thesis was to obtain information about the cellular composition of the epidermis and processes influencing and regulating epidermal growth and differentiation using novel cytometric techniques. Cytometry is defined here as the characterization and measurement of cells and cellular constituents for scientific, diagnostic and therapeutic purposes.

The methodology can be divided in three major categories. These are the use of monoclonal antibodies, cell cycle kinetics and the biological application of fluorescent indicators.

i) Use of monoclonal antibodies

Firstly, the use of antibodies, in particular monoclonal antibodies, will be discussed. Antibodies are proteins produced by vertebrates as a defense against infection. Using antibody-containing sera (see below), it is possible to detect specifically immunoreactive molecules (antigens) for instance on tissue sections. Conventionally, antibodies are prepared by injecting (purified) antigen into an animal, usually a rabbit or a sheep, and, after the animal has responded by producing antibodies against the antigen, collecting the antibody-containing serum. This antiserum is a mixture of different antibodies recognizing various parts of the antigen and will also contain antibodies cross reacting with other molecules than the antigen. It is this heterogeneity of antisera that made scientists search for new techniques for antibody preparation. In 1975 Köhler and Milstein published a report in which they describe the possibility of fusing antibody-producing B cells with rapidly growing cultured mouse myeloma cells[12]. Cells from the resulting heterogeneous mixture of hybrid cells, which are rapidly growing and producing antibodies, can be

cloned by limiting dilution. Such a hybridoma cell line, derived from a single B cell and producing an unique antibody (monoclonal antibody), provides a permanent and stable source of antibody. The advantages of monoclonal antibodies over convential antisera are clearly their uniform specificity (only one antigen binding site) and that they offer in principle an unlimited supply of antibody. Furthermore, unpurified antigen can be used for immunization because individual hybridoma clones, producing a particular antibody, can be selected afterwards. This technique made it possible that, in principle, antibodies can be produced against any protein in a cell or tissue, making them versatile and sensitive tools for localizing and detecting specific biological molecules. In this thesis, intermediate filaments[13,14,15] and other differentiation-associated structural proteins in the cytoplasm[16], and proliferation-associated nuclear antigens[17,18] have been detected by the use of specific antibodies. Although this method seems straightforward, an antibody recognizing an antigen, careful attention is of importance not only with respect to technical factors such as cross-reactivity of the antibody (see above), but also with respect to masking of the antigen. In the case of cell biological applications antibody binding is generally visualized using indirect immunofluorescence and immunoperoxidase staining[19,20]. A biochemical method, such as protein blotting after gel electrophoresis, was not selected despite the fact that the technique is easy to perform, is sensitive and enables one to specifically detect single proteins in crude mixtures[21]. Instead, immunofluorescence and immunoperoxidase staining were the techniques of choice because the localization of the antigen is not lost and because these techniques can be used for phenotyping of individual cells in heterogeneous cell populations.

ii) Cell cycle kinetics

The second method used was cell cycle kinetics. Cell cycle kinetics can be studied with a number of techniques. Careful attention to methodology is of primary importance because many of the controversies found in the field of epidermal cell kinetics, some of which are shown in table I at p. 20, can be traced as pure methodological discrepancies.

Measurement of the incorporation of [^{3}H] thymidine into DNA

The incorporation of [^{3}H] thymidine into DNA is widely used as a measure of DNA synthesis and cell proliferation, especially *in vitro*. The main reason for

this technique to be so very popular is the ease of measurement of radioactivity with the use of liquid scintillation counters without the need of further equipment. Furthermore, it is generally believed that [^{3}H] thymidine is incorporated selectively into DNA and that it is a functional marker. Because of these criteria the technique is very often used as the only and final measurement of proliferative activity. However, there are several potential artifacts due to [^{3}H] thymidine incorporation. Firstly, [^{3}H] thymidine has to be metabolized and experimental conditions should not interfere with the activity of rate-limiting enzymes of thymidine metabolism. Davison and co-workers have shown that, when the growth of human keratinocytes was studied *in vitro*, the use of incorporation of [^{3}H] thymidine was limited as an indicator of keratinocyte proliferation. Changes in [^{3}H] thymidine incorporation failed to correlate with direct counts of cell number and measurement of DNA content because of a decrease in thymidine kinase activity during the proliferative growth phase[22]. One other potential pitfall which should be mentioned with respect to epidermal proliferation is the occurrence of repair synthesis, for example after UV irradiation, which can interfere with DNA synthesis due to epidermal growth.
Finally, there are two other potential pitfalls which apply to the technique in general. Firstly, [^{3}H] thymidine can be incorporated into macromolecules other than DNA and secondly, it can be degradated prior to incorporation into DNA. These last two examples and most other potential pitfalls and artifacts in the use of [^{3}H] thymidine for measurement of cell proliferation *in vitro* and *in vivo* have been reviewed by Maurer[23]. He concludes that, considering all the possible artifacts arising from the use of [^{3}H] thymidine, the disadvantages prevail and that other methods should be preferred for measuring cell proliferation rates.

Proliferative indices

These are numerical values of the fraction of epidermal cells occupying any phase of the cell cycle; formerly, only two of these were measurable, the mitotic index and the pulse labelling index, the latter usually measured by autoradiography after exposure of the skin to [^{3}H] thymidine for a relatively short period.

The mitotic index is simply the proportion of the cell population in mitosis at any time, and is derived by observation of skin sections under the micros-

cope. However, despite the simplicity of counting, the definition of the target cell population can readily modify the magnitude of the count. In this respect the definition of the germinative compartment in the epidermis can be problematical and has led to consequent large disparities in the mitotic indices from investigator to investigator[28,30,24]. In particular when normal epidermis is evaluated for mitotic indices an additional problem exists. Because proliferative activity is very low mitotic figures are very rare. In order to obtain statistical reliable values large numbers of sections have to be evaluated.

The pulse labelling index is the other useful and relatively easily measured proliferative index and gives the proportion of cells which are in S-phase. Until recently [^{3}H] thymidine and autoradiography had to be used to detect the labelled S-phase cells in skin sections. The main difficulty with this method is the necessity of [^{3}H] thymidine administration *in vivo* in humans. *Ex vivo* administration can introduce changes in tissue behaviour due to lack of growth substances in the incubation medium or induction of factors interfering with epidermal growth. Furthermore, the same problems as described with the use of mitotic indices dc also occur with this proliferative index. Recently, other thymidine analogues such as bromodeoxyuridine and iododeoxyuridine have been introduced and are preferred over [^{3}H] thymidine, especially when used in conjunction with flow cytometry (see p. 20).

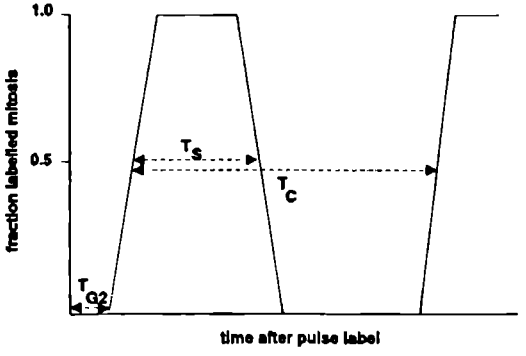


Figure 3 A hypothetical presentation of a FLM curve from which T_{G2}, T_S and T_C can be calculated.

The fraction of labelled mitosis (FLM) technique

The FLM technique is one of the earliest and one of the most informative cell cycle kinetic techniques. As with the proliferative index measurements tissue or cell samples have to be pulse labelled with [^{3}H] thymidine and fixed

at time intervals of 1-2 hours during a period of at least the expected cell cycle time. The fraction of labelled mitosis is counted by autoradiography of histological sections. A theoretical FLM curve is shown in figure 3. From such a curve duration of G_2 (T_{G2}), S (T_S) and the total cell cycle time (T_C) can be calculated[25]. When this technique is applied to biological material results are not always as ideal as suggested in figure 3. For hyperproliferative epidermis from psoriatic patients Weinstein was able to construct an acceptable FLM curve[26]. A value for normal epidermis has not been published so far probably because of reasons already discussed at p. 18. A main disadvantage of the use of this technique *in vivo* is again that radioactivity has to be used. Furthermore, a follow up of 48 h with a 2 h time interval results in 24 skin samples to be taken. This will be at least inconvenient for most volunteers.

Continuous labelling

This technique also exploits [^{3}H] thymidine but in a rather different way. Instead of making the label available for only a short period (pulse labeliing) the cell population under study is subjected to the DNA precursor continuously for a long period. In this way it is claimed that the growth fraction can be measured. This can even be achieved *in vivo*[27]. The only data obtained from human skin *in vivo* were published by Gelfant[28]. In this study skin samples were taken from one cancer patient with normal appearing untreated skin who had been infused with [^{3}H] thymidine continuously for 8½ days to study cell kinetics in multiple myeloma. The technique of continuous labelling is probably best suited to use *in vitro* where the label can be added without perturbing the culture. Samples of the cell population under study are removed and fixed at time points which will depend upon the characteristics of the particular cell population but will mostly be taken at a time point equal or later than the expected duration of the cell cycle. However, this method will only work when culture conditions nor cell behaviour change during the incubation period with the DNA precursor. Where in the past [^{3}H] thymidine was the only available DNA precursor suited for this technique, in recent experiments bromodeoxyuridine incorporation and its detection by an antibody is often preferred over the use of the radioactive label (see chapter VII).

Table I Overview of *in vivo* cell kinetic data in normal (N) and psoriatic involved (PI) epidermis showing some of the controversies found in the field of epidermal cell kinetics.

Tissue	LI[a]	%S (FCM)[b]	T_S (h)[c]	T_C (h)[d]	Reference
N	2.2				Lachapelle & Gillman[29]
N	3.5		16	457	Weinstein[30]
N	5.8		10.3	184	Heenen et al[31]
N	3.8		7.6	206	Allegra & De Panfilis[32]
N		3.0			Frenz et al[33]
N		2.0	10	36-60	Bauer[3]
N	2.7		14	311	Weinstein et al[34]
PI	22.7		8.5	37.5	Weinstein & Frost[35]
PI	11.0		9.8	91	Goodwin et al[36]
PI	10.3		7.7	56	Duffill et al[37]
PI	21.2				Gelfant et al[38]
PI		9.0		40-60	Bauer[3]

[a]LI = pulse labelling index
[b]%S (FCM) = fraction of S-phase cells measured by flow cytometry
[c]T_S (h) = duration of S-phase
[d]T_C (h) = total cell cycle time

Flow cytometry

The technical principles of flow cytometry (FCM) are dealt with below. FCM has been more and more used for cell kinetic studies during the last two decades. In its simplest form suspended individual cells are stained with a fluorescent dye such as ethidium bromide or propidium iodide. DNA specific fluorescence per cell is measured, plotted as a histogram and analyzed (for an example see chapter II, figure 7). From these histograms percentage diploid cells (cells in G_0/G_1 or differentiated), percentage S-phase cells and percentage cells with tetraploid DNA content can be calculated using a number of more or less sophisticated methods[39,40].

For analysis of epidermal cell cycle kinetics Baisch's graphical method is frequently used[41]. This method is developed for analysis of DNA histograms with a low flat S region and clearly defined G_1 and G_2M peaks. All the S-phase cells are assumed to fill a rectangle between the G_1 and G_2M peak modals, the height of the rectangle being the average cell number in the mid S region.

The fraction of cells in the G_1 and G_2M-phases are calculated by subtraction of the rectangular S distribution.

When DNA distributions of kinetically perturbed populations have to be analyzed other methods should be selected which attempt to calculate the S-phase fraction by a curve fitting approach[39]. A modification of the curve fitting technique was published in 1980. This simplified method replaces the S-phase region by a second degree polynomial. The S-phase fraction is then calculated by fitting to that part of the distribution, mid S-phase, which is not influenced by either the G_1 or the G_2M peak[42]. The function is extrapolated to the G_1 and G_2M peak modals and the S-phase fraction is identified by the area between these limits. It should be noted that cell kinetic studies, however, do not specifically indicate that the S-phase distribution has the form of a second degree polynomial This assumption is made in the absence of better information

Instead of polynomial fit procedures of the S-phase region (with Baisch's method the S-phase region is in fact fitted with a zero degree polynomial), sometimes more complex methods of analysis are preferred For instance, a series of normal curves can be used to fit the entire distribution, with the first and last curves representing the G_1 and G_2M-phases, respectively. The normal curves of the S-phase region are each representing a number of cells in a certain part of the S-phase. Different techniques are available for calculating the parameters of the normal curves In contrast to the above-mentioned methods, these techniques require additional input data in order to obtain a reliable and "reasonably looking" solution[43]

From comparative studies of Dean and co-workers some important conclusions can be drawn with respect to the reliability and interpretation of DNA distribution analysis[40,44] Generally, all methods tested (including the ones described above) yielded reasonably accurate fractions of cells in each phase with relative errors in the range of 10-20%, depending on type of cells and the DNA-specific dye used The errors are biggest in calculation of the G_2M-phase and most methods overestimate the G_1 fractions A small coefficient of variance (CV) automatically results in a more accurate analysis and relatively simple methods to analyze DNA distributions (Baisch's method and simplified method of Dean) require CVs < 7. Finally, in order to obtain ac-

curate S-phase fraction estimates flow cytometric data should be compared with corresponding pulse labelling indices.

Because of the heterogeneity of the diploid cell population, Darzynkiewicz and co-workers developed a two-colour fluorescence measurement using one dye, acridine orange, staining in each cell DNA and RNA[45]. This technique allows further analysis of cells in G_0 and G_1. It, however, could not be adapted for use with human epidermal keratinocytes (Bauer FW, unpublished observations). By analogy with [^{3}H] thymidine incorporation, a recently developed method to label S-phase cells selectively is based on the incorporation of the thymidine analogue bromodeoxyuridine into DNA during S-phase. Details on this method, with special reference to epidermal cells, are discussed in chapter III. The main advantage of the use of bromodeoxyuridine (or iododeoxyuridine) over the use of [^{3}H] thymidine is that the non-radioactive thymidine analogues can be administrated *in vivo* in humans. Furthermore, flow cytometric analysis of bromodeoxyuridine incorporation and DNA content allows assessment of the number of cells in S-phase (comparable to the pulse labelling index) and the duration of the S-phase (T_S) even from one single sample[46].

iii) The biological application of fluorescent indicators

A third method was the biological application of fluorescent indicators. Where the use of fluorescein isothiocyanate (FITC) for labelling antibodies and other molecules and quantitative DNA stains such as propidium iodide (PI), ethidium bromide, mithramycin, DAPI (4'-6-diamidino-2-phenylindole) and acridine orange are more or less standard in cytometry, other applications are relatively limited. There are large gaps in the literature when one looks for information about the biological applications of fluorescence and fluorescent dyes. As Howard Shapiro states in his book on practical flow cytometry: "I previously threatened to write a book on fluorescence and fluorescent dyes and their biological applications; if I don't decide that sex and violence will sell better, I may still do that."[47] One additional application worked out in this thesis is the use of pH-dependent fluorescent dyes for measurement of intracellular pH[48]. In principle, using the same methodology, it is also possible to measure other functional characteristics of individual cells, such as calcium-ion concentrations, membrane potential, and enzyme activities. There is some

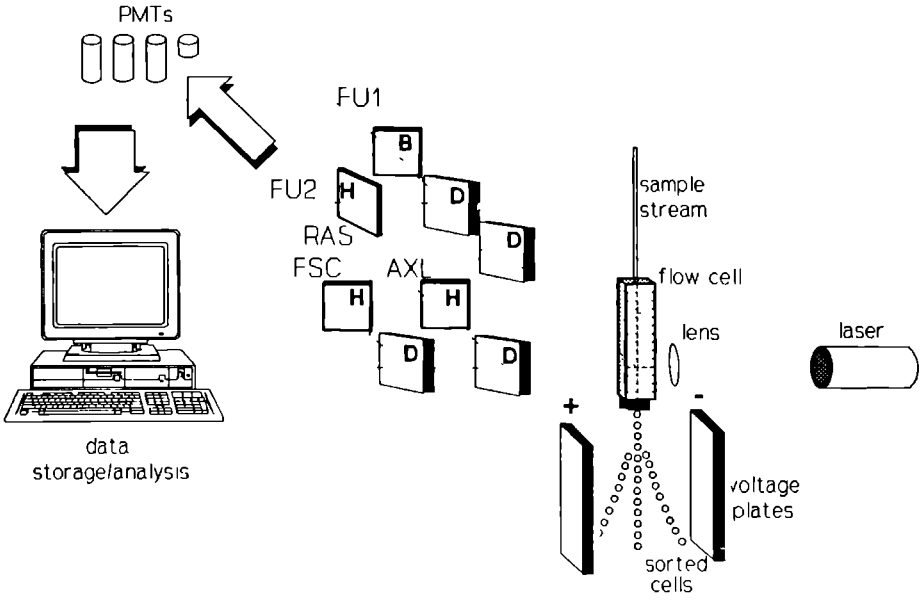


Figure 4. Schematic drawing of the FCM system as it is used at our department. (PMT=photomultiplier; FU1 and FU2 are fluorescent signals; RAS=right angle scatter, FSC=forward scatter, AXL=axial light loss)

overlap between the different methods in that fluorescent indicators can be utilised to label antibodies, antibodies can be used to define the target population and fluorescent indicators can be applied to stain DNA in cell kinetic studies.

FCM and immunohistochemistry are the techniques used to analyze the different cell types and processes of the epidermis. The principle of FCM is that cells, or other biological particles (virus particles, liposomes, cellular organelles, bacteria and fungi, chromosomes, cell hybrids, tumour cell spheroids) in aqueous suspension are made to flow at high speed through a sensing region where optical and electrical signals, indicative for biological properties are generated[49,50]. These signals are stored and analyzed for quantitative evaluation. The light source is generally a laser or an arc lamp, capable of exciting fluorescent emission. Each cell receives uniform illumination and generates a burst of scattered light. If a fluorochrome is coupled to the cells, a burst of fluorescence light is generated over all angles. Fluorescent light emission can be separated from the scattered light by means of its

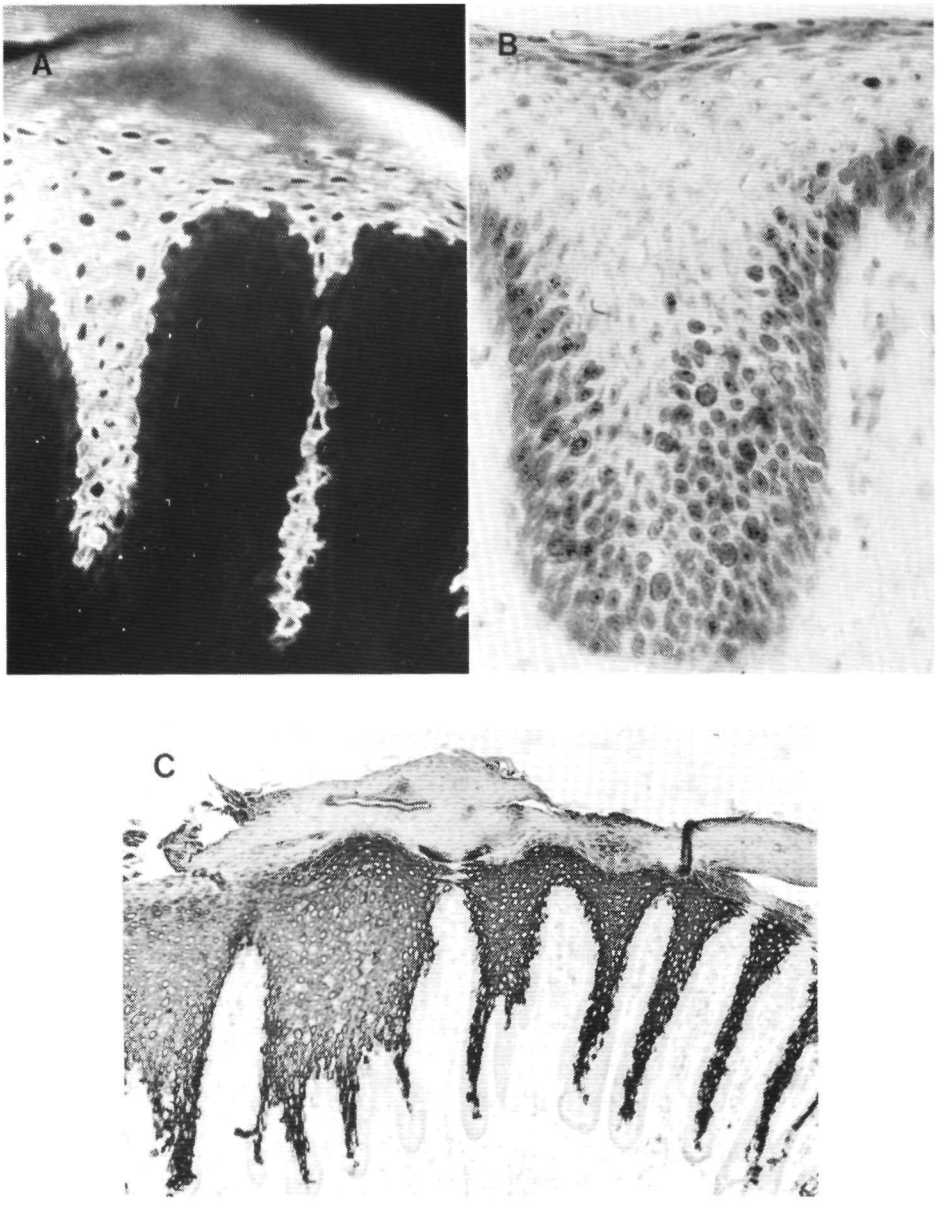


Figure 5. Immunofluorescent labelling with RKSE60 (A) and immunoperoxidase labelling with Ki-67 (B) and RKSE60 (C) in psoriatic skin.

Tabel II Major cellular parameters measurable by FCM. The parameters are divided into two groups, depending upon whether they can or cannot be measured without use of a reagent.

INTRINSIC	EXTRINSIC
cell size	DNA content
cell shape	RNA content
cytoplasmic granularity	total protein
pigment content (e.g. haemoglobin, lipofucsins)	sulfhydryl groups
	antigens
	surface glycoproteins (e.g. lectin binding)
	membrane permeability
	enzyme activity
	phagocytic activity

greater wave-lengths and can be measured independently and simultaneously for each cell. The scatter signal contains information about volume and shape of the particle. A list with major parameters (physical and chemical characteristics) which can now be measured by FCM is given in table II. The FCM configuration as used at our department is schematically drawn in figure 4.

Using immunofluorescence and immunoperoxidase techniques applied to cryostat sections, only qualitative or semi-quantitative information can be obtained about different cell types in the epidermis and their relation to processes such as proliferation and differentiation (figure 5). However, most of the morphology and hence the location of certain cell types is maintained. Therefore FCM and immunohistochemistry are complementary techniques, which together permit qualitative and quantitative population analysis, extending our knowledge of topographic relations in the epidermis as visualized by classical light microscopy.

7. References

1. Mier PD, Cotton DWK: The Molecular Biology of Skin. Blackwell Scientific Publications, Oxford, pp 3, 1976

2. The theory of renewing cell populations. in: The biology of epithelial cell populations, Volume 1. Eds. Wright N, Alison M, Clarendon Press, Oxford, pp 22-96, 1984

3. Bauer FW: Cell kinetics. In: Textbook of Psoriasis. Eds. Mier PD, Van de Kerkhof PCM, Churchill Livingstone, Edinburgh, pp 100-112, 1986

4. Eckert RL, Rorke EA: Molecular biology of keratinocyte differentiation. Environ Health Perspect 80:109-116, 1989

5. Pinkus H: Examination of the epidermis by the strip method of removing horny layers. I. Observation on thickness of the horny layer and on mitotic activity after stripping. J Invest Dermatol 16:383-386, 1951

6. Boezeman JBM, Bauer FW, de Grood RM: Flow cytometric analysis of the recruitment of G_0 cells in human epidermis in vivo following tape stripping. Cell Tissue Kinet 20:99-104, 1987

7. Van de Kerkhof PCM: Clinical features. in: Textbook of Psoriasis. Eds. Mier PD, Van de Kerkhof PCM, Churchill Livingstone, Edinburgh, pp 13-39, 1986

8. Rheinwald JG, Green H: Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331-343, 1975

9. Watt FM, Green H: Stratification and terminal differentiation of cultured epidermal cells. Nature 295:434-436, 1982

10. Watt FM, Mattey DL, Garrod DR: Calcium-induced reorganization of desmosomal components in cultured human keratinocytes. J Cell Biol 99:2211-2215, 1984

11. Adams JC, Watt FM: Fibronectin inhibits the terminal differentiation of human keratinocytes. Nature 340:307-309, 1989

12. Köhler G, Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495-497, 1975

13. Woodcock-Mitchell J, Eichner R, Nelson WG, Sun T-T: Immunolocalization of keratin polypeptides in human epidermis using monoclonal antibodies. J Cell Biol 95:580-588, 1982

14. Van Muijen GNP, Warnaar SO, Ponec M: Differentiation-related changes of cytokeratin expression in cultured keratinocytes and in fetal, newborn, and adult epidermis. Exp Cell Res 171:331-345, 1987

15. Weiss RA, Eichner R, Sun T-T: Monoclonal antibody analysis of keratin expression in epidermal diseases: a 48- and 56-kdalton keratin as molecular marker for hyperproliferative keratinocytes. J Cell Biol 98:1397-1406, 1984

16. Murphy GF, Flynn TC, Rice RH, Pinkus GS: Involucrin expression in normal and neoplastic human skin: a marker for keratinocyte differentiation. J Invest Dermatol 82:453-457, 1984

17. Gratzner HG: Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. Science 218:474-475, 1982

18. Gerdes J, Schwab U, Lemke H, Stein H: Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31:13-20, 1983

19 Ramaekers FCS, Puts JJG, Moesker O, Kant A, Huysmans A, Haag D, Jap PHK, Herman CJ, Vooijs GP. Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumours an overview Histochem J 15 691-713, 1983

20 Bauer FW, Boezeman JBM, van Engelen L, de Grood RM, Ramaekers FCS· Monoclonal antibodies for epidermal population analysis J Invest Dermatol 87 72 75, 1986

21 Beisiegel U Protein blotting Electrophoresis 7 1 18, 1986

22 Davison P, Liu S, Karasek M Limitations in the use of [^{3}H] thymidine incorporation into DNA as an indicator of epidermal keratinocyte proliferation *in vitro* Cell Tissue Kinet 12 605-614, 1979

23 Maurer HR Potential pitfalls of [^{3}H] thymidine techniques to measure cell proliferation Cell Tissue Kinet 14 111-120, 1981

24 Van Scott EJ, Ekel TM Kinetics of hyperplasia in psoriasis Arch Dermatol 88 373-381, 1963

25 Steel GG, Hanes S The technique of labelled mitosis analysis by automatic curve fitting Cell Tissue Kinet 4 93-105, 1971

26 Weinstein GD Epidermal cell kinetics in Dermatology in general medicine Eds Fitzpatrick TB, Eisen AZ, Wolff K, Freedberg IM, Austen KF, McGraw Hill book company, New York, pp 154-165, 1987

27. Denekamp J, Stewart FA, Douglas BG Changes in the proliferation rate of mouse epidermis after irradiation continuous labelling studies Cell Tissue Kinet 9 19 29, 1976

28 Gelfant S On the existence of non cycling germinative cells in human epidermis *in vivo* and cell cycle aspects of psoriasis Cell Tissue Kinet 15 393-397, 1982

29 Lachapelle JM, Gillman T Tritiated thymidine labelling of normal human epidermal nuclei A comparison, in the same subjects of *in vivo* and *in vitro* techniques Brit J Dermatol 81 603-616, 1969

30 Weinstein GD Biochemical and pathophysiological rationale for methotrexate in psoriasis Ann New York Acad Sci 186 452-466, 1971

31 Heenen M, Achten G, Galand P Autoradiographic analysis of cell kinetics in human normal epidermis and basal cell carcinoma Cancer Res 33 123-127, 1973

32 Allegra F, De Panfilis G An in vivo method of studying the kinetics of cell proliferation in normal human epidermis Acta Derm Venereol (Stockh) 54 87 90, 1974

33 Frenz G, Möller U, Christensen I DNA flow cytometry on human epidermis 1 Methodological studies on normal skin J Invest Dermatol 74 119 121, 1980

34 Weinstein GD, Ross P, McCullough JL, Colton A Proliferative defects in psoriasis in Psoriasis cell proliferation Eds Wright NA, Camplejohn RS Churchill Livingstone, Edinburgh, pp 189-208, 1983

35 Weinstein GD, Frost P Abnormal cell proliferation in psoriasis J Invest Dermatol 50 254-259, 1968

36. Goodwin P, Hamilton S, Fry L: The cell cycle in psoriasis. Brit J Dermatol 90:517-524, 1974

37. Duffill M, Wright N, Shuster S: The cell proliferation kinetics of psorlasis examined by three in vivo techniques. Brit J Dermatol 94:355-362, 1976

38. Gelfant S, Ozawa A, Chalker DK, Smith JG: Circadian rhythms and differences in epidermal and in dermal cell proliferation in uninvolved and involved psoriatic skin *in vivo*. J Invest Dermatol 78:58-62, 1982

39. Dean PN: Quantitative analysis of flow systems data. In: Pulse-Cytophotometry. ed. Lutz D, European Press, Ghent, Part III, pp 63-69, 1978

40. Baisch H, Beck H-P, Christensen IJ, Hartmann NR, Fried J, Dean PN, Gray JW, Jett JH, Johnston DA, White RA, Nicolini C, Zeitz S, Watson JV: A comparison of mathematical methods for the analysis of DNA histograms obtained by flow cytometry. Cell Tissue Kinet 15:235-249, 1982

41. Baisch H, Göhde W, Linden WA: Mathematical analysis of ICP-data to determine the fraction of cells in the various phases of cell cycle. In: Pulse-Cytophotometry. eds. Haanen CAM, Hillen HFP, Wessels JMC, European Press, Ghent, Part I, pp 68-76, 1975

42. Dean PN: A simplified method of DNA distribution analysis. Cell Tissue Kinet 13:299-308, 1980

43. Van der Linden, PM: Computer analysis of DNA histograms. Thesis, Faculty of Medicine, Utrecht, 1980

44. Dean PN, Gray JW, Dolbeare FA: The analysis and interpretation of DNA distributions measured by flow cytometry. Cytometry 3:188-195, 1982

45. Darzynkiewicz Z, Sharples T, Staiano-Coico L, Melamed MR: Subcompartments of the G_1 phase of cell cycle detected by flow cytometry. Proc Natl Acad Sci USA 77:6696-6699, 1980

46. Begg AC, McNally NJ, Shrieve DC, Kärcher H: A method to measure the duration of DNA synthesis and the potential doubling time from a single sample. Cytometry 6:620-626, 1985

47. Shapiro HM: Practical Flow Cytometry. Alan R. Liss, Inc., New York, pp 9, 1988

48. Graber ML, Dilillo DC, Bradford LF, Pastoriza-Munoz E: Characteristics of fluoroprobes for measuring intracellular pH. Anal Biochem 156:202-212, 1986.

49. Bauer FW, Boezeman JBM: Flow cytometric methods in human skin with respect to cell cycle kinetics. in: Psoriasis: cell proliferation. Eds. Wright NA, Camplejohn NS, Churchill Livingstone, Edinburgh, pp 104-116, 1983

50. Shapiro HM: Practical Flow Cytometry. Alan R. Liss, Inc., New York, pp 1-86, 1988

CHAPTER II. INTERMEDIATE FILAMENTS AND DIFFERENTIATION-RELATED PROTEINS AS COMPARTMENT SPECIFIC MARKERS IN THE EPIDERMIS

Parts of this chapter were based on the following publications:

Saskia de Mare, Piet EJ van Erp, Peter CM van de Kerkhof, Epidermal hyperproliferation assessed by the monoclonal antibody Ks8.12 on frozen sections. J Invest Dermatol 92:130-131, 1989

Piet EJ van Erp, Joris J Rijzewijk, Jan BM Boezeman, John Leenders, Saskia de Mare, Peter CM van de Kerkhof, Frans CS Ramaekers, Franz W Bauer, Flow cytometric analysis of epidermal subpopulations from normal and psoriatic skin using monoclonal antibodies against intermediate filaments. Am J Pathol 135:865-870, 1989

Saskia de Mare, Piet EJ van Erp, Frans CS Ramaekers, Peter CM van de Kerkhof, Flow cytometric quantification of human epidermal cells expressing keratin 16 in vivo after standardized trauma. Arch Dermatol Res 282:126-130, 1990

CHAPTER II. INTERMEDIATE FILAMENTS AND DIFFERENTIATION-RELATED PROTEINS AS COMPARTMENT SPECIFIC MARKERS IN THE EPIDERMIS

1. Introduction 31
2. Methods & Materials 33
2.1. Skin Sampling (33)
2.2. Preparation of Cell Suspensions (33)
2.3. Cell Culturing (33)
2.4. Immunohistochemical Staining (34)
2.5. Staining of cell suspensions (35)
2.6. Flow cytometry (35)
3. Results and Discussion 36
4. References 44

1. Introduction

Epidermis is a continuously renewing epithelium. Cells from the basal layer divide and as they leave this layer and begin migrating outward to the cornified layers they undergo a series of morphological and biochemical changes. By finally desquamating from the stratum corneum, a dynamic equilibrium is maintained between cell loss and cell renewal. The mechanisms by which this process is controlled are only partially understood.

One of the changes of normal, terminally differentiating keratinocytes is the specific expression of keratin-type intermediate filaments. While basal cells express two major keratins, a 58kD protein (keratin 5) and a 50kD protein (keratin 14), suprabasal cells express four other keratins, proteins of molecular weight 68kD (keratin 1), 65.5kD (keratin 2), 56.5kD (keratin 10) and 56kD (keratin 11)[1,2,3,4]. In cultured human keratinocytes, keratins of molecular weight 59kD, 56kD, 54kD, 48kD, 46kD, and 40kD (keratins 4, 6, 13, 16, 17 and 19, respectively)[5,6,7] and in diseased skin keratins 4, 6, 16 and 17[3,8,9,10] are additionally synthesized, while the expression of the differentiation-associated keratins can be suppressed. In contrast to the differential expression of

keratins in epithelial cells, cells of mesenchymal origin, such as melanocytes and Langerhans cells are characterized by the expression of a single intermediate filament-type protein, vimentin[11]. Additional to the intermediate filament-type proteins, other structural proteins are synthesized in considerable amounts, especially in relation to the formation of the cornified envelopes. A well-known example of such a protein is the cornified envelope precursor protein involucrin, which is specifically expressed in keratinocytes from the upper layers of the epidermis[12]. Other proteins, such as the keratohyalin-related proteins profilaggrin and filaggrin are related to differentiation-specific organelles[13]. Proper and accurate identification of epidermal subpopulations expressing specific structural or intermediate filament-type proteins should lead to a better understanding of the complex mechanisms by which epidermal growth control is achieved[14].

Several methods for detection of the intermediate filament-type proteins are available. Two-dimensional polyacrylamide gel electrophoresis is the technique most often used to characterize keratin proteins biochemically[3,9]. The advantage of this method is the specific detection of the whole repertoire of keratins present in a tissue specimen. However, a limitation of this rather elaborate method is that information on the localization of the keratins is lost. In recent years MAb recognizing sets of related keratins, or even recognizing single keratins, have been developed and are becoming commercially available[4,15,16]. In addition specific antibodies directed against involucrin and filaggrin became available. Extensive studies have established the usefulness of these antibodies as cell-type markers in histology and tumour diagnosis, and in studying growth and differentiation of epithelial cells. This immunohistochemical method has the advantage that topographical information is maintained. However, the approach is time consuming and only qualitative or semi-quantitative information can be obtained.

In this chapter we report on two approaches: firstly, immunohistochemistry on cryostat sections and alternatively, flow cytometric quantification of intermediate filament-type proteins in single cell suspensions of epidermal cells. FCM allows rapid simultaneous quantification of two or more characteristics per individual cell with high reproducibility and statistical accuracy. The primary aim of the investigation was the development of a flow cytometric technique to

analyze both DNA content and binding of MAb against intermediate filaments in single cells. Furthermore, immunohistochemistry was used to obtain topographical information and to test the specificity of the antibodies.

2. Methods & Materials

2.1. Skin Sampling

Skin specimens were obtained by shave biopsy (about 0.2 mm thick, 3 mm diameter) using a razor blade in conjunction with a metal guard[17] from normal skin of healthy volunteers, clinically uninvolved skin and lesional skin of patients with psoriasis. Healthy controls and patients were aged between 18 and 64 years. In general, biopsies were taken from the back and in the case of psoriatic uninvolved skin at least 10 cm from the margin of the nearest lesion. During at least 2 weeks before biopsies were taken, patients discontinued their topical medication and no systemic anti-psoriatic medication had been taken during the last year. Furthermore, biopsies were taken from sello-tape-stripped sites at the back of healthy volunteers at different time intervals after stripping[18].

2.2. Preparation of Cell Suspensions

Cell suspensions were prepared by trypsinization of the biopsies, as described previously[19]. Stratum corneum and the dermis remained intact during this procedure, yielding a single cell suspension with little admixture of dermal cells. Cells were fixed in ice-cold 70% ethanol and stored at -20°C until use.

2.3. Cell Culturing

Human neonatal foreskin keratinocytes were cultured on 3T3 feeder cells[20]. Epidermal cells were seeded on lethally irradiated (3000 Rad in 3 min) Swiss mouse 3T3 fibroblasts in DMEM/F12 3:1 v/v (Flow Laboratories, Irvine, Scotland) supplemented with 0.4 μg/ml hydrocortisone (Collaborative Research Inc., Lexington, MA, USA), 10^{-6}M isoproterenol (Sigma, St.Louis, MO, USA), 100 U/ml penicillin plus 100 μg/ml streptomycin (Gibco, Breda, The Netherlands), 5% v/v fetal bovine serum (Seralab, Nistelrode, The Netherlands) and, starting at day three after seeding with 10 ng/ml epidermal growth factor (Collaborative Research Inc., Lexington, MA, USA). Cells were grown at 37°C, 95% relative humidity, and 7.5% CO_2 in air. In some experiments keratinocytes were grown on thermonox acetone-resistant plastic coverslips. EDTA treated,

trypsinized keratinocytes or coverslips with keratinocyte colonies from the primary culture or from the first 1-2 passages were used in the experiments.

2.4. Immunohistochemical Staining

Biopsies were frozen immediately in Tissue-Tek II OCT compound (Miles Scientific, Napperville, USA) and sectioned at 6-8 μm. After fixation with acetone, the frozen sections were incubated for 3 min in PBS containing 0.01% Tween 80. Coverslips with acetone-fixed keratinocytes were mounted onto slides with DePeX. After washing with PBS, slides were incubated for 30 min at room temperature with primary antibody in a proper dilution (table I, list of antibodies used). After 3 washes with PBS the slides were incubated for 30 min at room temperature with peroxidase-conjugated rabbit anti-mouse immunoglobulins (RAMPO, Dakopatts, Copenhagen, Denmark) diluted 1:25 in PBS containing 5% human AB-serum, or peroxidase-conjugated swine anti-rabbit immunoglobulins (SWARPO, Dakopatts, Copenhagen, Denmark) diluted 1:25 in PBS containing 5% human AB-serum. After three further washes with PBS and a preincubation in sodium acetate buffer, pH 4.9, slides were finally stained in sodium acetate buffer containing 10 mg/50 ml 3-amino-9-ethyl-carbazole (AEC) and 17.5 μl/50 ml 30% H_2O_2 (prepared freshly) for 10 min at room temperature in the dark. After two washes with demineralized water, slides were counterstained with haematoxylin (Mayer) and mounted in glycerin gelatin.

The antibodies used are given in table I. These comprize RKSE60, RCK102 and K40 (produced at the department of Pathology, University Hospital, Nijmegen), Pab601 (kindly provided by Dr I. Leigh, London), K_s8.12 (Bio-Yeda, Rehovot, Israel and at a later stage Sigma, St. Louis, USA), MVI (Eurodiagnostics, Apeldoorn, The Netherlands), anti-involucrin and anti-filaggrin (Biomedical Technologies, Inc, Stoughton, USA). RKSE60 is directed against keratin 10 and recognizes only suprabasal keratinocytes[16]. RCK102 reacts with keratins 5 and 8 and has comparable staining characteristics to Pab601 binding to cells of the basal layer in normal epidermis, and cells in the lowest three layers of the epidermis in psoriatic lesional skin. Although the specificity of Pab601 with respect to epidermis has not yet been established as far as we know, it is believed to recognize keratins 5 and/or 14[21]. K40 is a polyclonal antibody having a broad specificity and recognizing all keratino-

cytes in cryostat sections of normal and diseased skin and in cultures. K_s8.12 binds to keratin 16 present in the suprabasal compartment of hyperproliferative epidermis[22]. The polypeptide specificity of K_s8.12 was examined by immunoblotting analysis of cytoskeletal preparations containing nearly all human keratin polypeptides and reacted only with keratins 13 and 16[15]. MVI is an anti-vimentin MAb[16], staining non-keratinocytes in the epidermis. Involucrin is a precursor protein of the cornified envelope, and anti-involucrin stains the upper layers of normal epidermis. In psoriatic lesional epidermis all suprabasal layers are positively stained. Anti-filaggrin stains the granular layer of the epidermis in a granular pattern. The specificity of the MAb is summarized in table I.

2.5. Staining of cell suspensions

Aliquots of ethanol-fixed single cell suspensions derived from single biopsies were immunostained according to Bauer et al[14]. Dilutions were as with cryostat sections and are given in table I. FITC-conjugated goat anti-mouse IgG (GAMFITC, Tago, Burlingame, USA) and FITC-conjugated swine anti-rabbit IgG (SWARFITC, Dakopatts, Copenhagen, Denmark) were added in a 1:25 dilution, as second antibodies. After the two incubation steps, the cells were washed twice and resuspended in 200 μl PBS. Cellular DNA was stained quantitatively by the addition of 200 μl sodium phosphate-buffer, pH 7.8 containing 4 mg/ml propidium iodide (PI) (Calbiochem, San Diego, USA).

2.6. Flow cytometry

The cell suspensions were incubated for 15 min with 50 μl RNAse (1 mg/ml in PBS) (Sigma, St Louis, USA) at room temperature, filtered in order to remove clumps and the fluorescence measured using an Ortho 50H flow cytometer (Ortho Instruments, Westwood, USA). Both FITC and PI were excited with a 5 W argon ion laser (164-05, Spectra Physics, Mountain View, USA) tuned at 488 nm and emission was recorded at 515-530 nm (FITC) and >630 nm (PI). The data were stored and analyzed with a Digital PDP11/34 computer (Digital Equipment, Galway, Republic of Ireland). Both area and peak value of the red (PI) fluorescence signal were measured. The ratio area/peak is an excellent discriminator between artifacts due to doublets of diploid cells and real single tetraploid cells[19].

Table I List of monoclonal antibodies used in this study and their demonstrated specificity.

NAAM	BIOCHEMICAL SPECIFICITY	LOCALIZATION IN NORMAL EPIDERMIS	LOCALIZATION IN PSORIATIC LESION	LOCALIZATION IN VITRO	DILUTION	REF.
RKSE60	keratin 10	suprabasal layers	suprabasal layers	sporadically	1:10	[16]
RCK102	keratins 5 and 8	basal layer	lowest three layers	all keratinocytes	1:20	
K40	most keratins	all layers	all layers	all keratinocytes	1:50	
Pab601	not established (keratins 5 and/or 14 ?)	basal layer	lowest three layers	all keratinocytes	1:2.5	[21]
K_s8.12	keratins 13 and 16	not present	suprabasal layers	keratinocytes, stronger staining for peripheral cells	1:20	[15] [22]
MVI	vimentin	non-keratinocytes	non-keratinocytes	human fibroblasts, some keratinocytes	undiluted	[16]
α-involucrin	involucrin	granular layer	suprabasal layers	10-30% of the keratinocytes	1:8	[12]
α-filaggrin	profilaggrin and filaggrin	granular layer	granular layer	2-10% of the keratinocytes	1:500	[13]

3. Results and Discussion

Intermediate filaments and other structural proteins such as involucrin and filaggrin can easily be visualized immunohistochemically using a standard immunoperoxidase protocol, because of the high antigen concentration per cell. Figure 1 shows an example of RKSE60 staining in a cryostat section of psoriatic lesional skin. Bright and intense staining of suprabasal cells illustrates that RKSE60, recognizing keratin 10, is a useful differentiation marker for epidermis. The same pattern can be seen in normal epidermis.

Even in the case of the hyperproliferation marker K_s8.12, where staining of

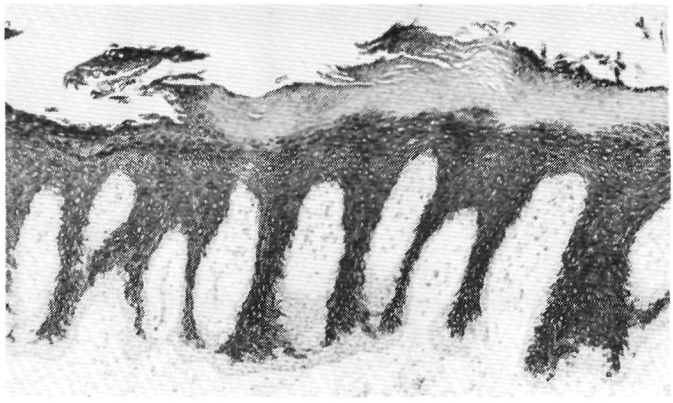


Figure 1 RKSE60 staining of cryostat section derived from psoriatic lesional skin, using an indirect immunoperoxidase technique.

normal skin was patchy, there was a sharp demarcation between positive and negative areas. The percentage of cells staining varied between healthy volunteers, but was minimal and always restricted to deeper rete ridges of the basal layer. Figure 2 demonstrates an example representative of all the healthy volunteers. The clinically uninvolved skin of psoriatic patients showed an essentially similar pattern to that observed in healthy volunteers.

In contrast, the lesional skin of all psoriatic patients showed a positive K_s8.12 staining of the suprabasal layers, which was more pronounced and uniformly distributed. These characteristics can be seen in figure 3.

Finally figure 4 illustrates the specific staining pattern of anti-filaggrin. The keratohyalin granules in the stratum granulosum can be clearly identified, indicating that this antibody is very useful as a marker of the very late stages of terminal differentiation.

For cultured human keratinocytes the same immunoperoxidase technique can be used as for cryostat sections obtained from skin. However, these keratinocytes were cultured in plastic petridishes. Because this plastic is not resistant against the acetone fixation step used with the immunoperoxidase technique, thermonox cover slips had to be used. Figure 5A and 5B show examples of staining with RCK102 and anti-vimentin, respectively.

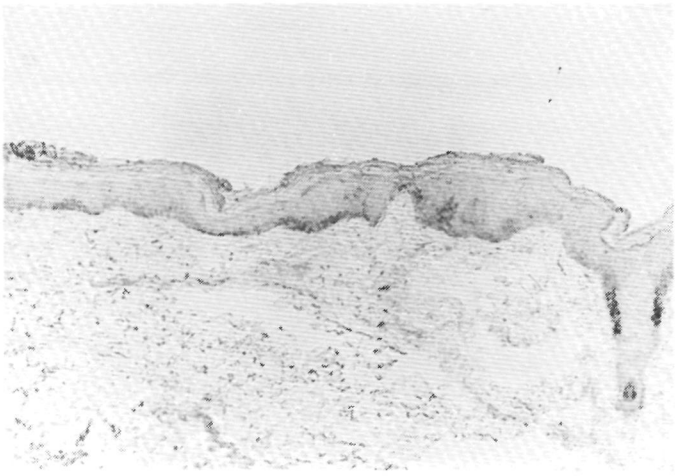


Figure 2 K_s8.12 staining in normal epidermis.

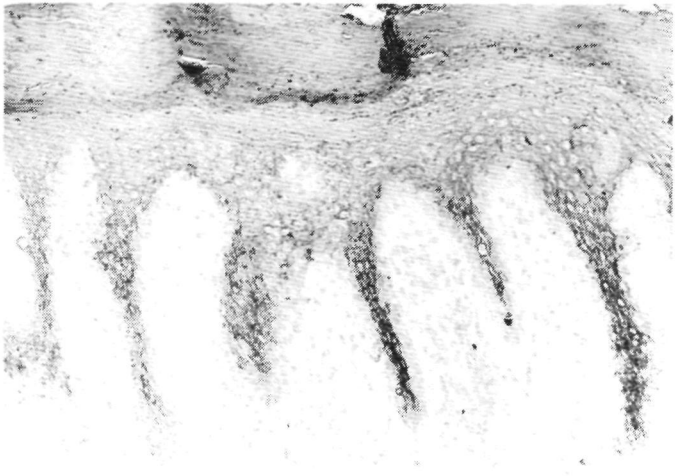


Figure 3 K_s8.12 staining in psoriatic lesional epidermis.

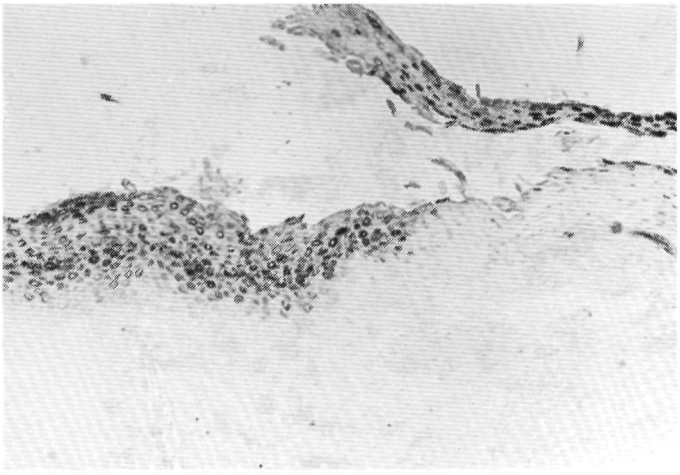


Figure 4 Typical staining pattern of psoriatic lesional epidermis using anti-filaggrin.

For flow cytometric analysis cell suspensions were fixed in 70% ethanol. Other fixatives, such as acetone and methanol, were also tested but resulted in cell loss, DNA histograms with larger coefficients of variance, and sometimes less antibody binding. Figure 6 illustrates a typical 2-parameter plot of normal epidermal cells stained with RKSE60 and PI. A cluster of FITC-positive cells with mainly low relative DNA content (diploid) is clearly separated from the FITC-negative cells which have relative DNA content varying from diploid to tetraploid. Figure 7 is a straight conversion of the original list data, resulting in a histogram of the red fluorescence signal (relative DNA content). In this particular example we found a percentage of cells in S+G2M phase of 5.7%. The average percentage of cells in S+G2M phase in normal epidermis is 4.0% ± 1.2 (st.dev; n=13). This value increases in epidermis of psoriatic lesions to an average of 12.5% ± 5.2 (n=16). These DNA values are identical to pre-

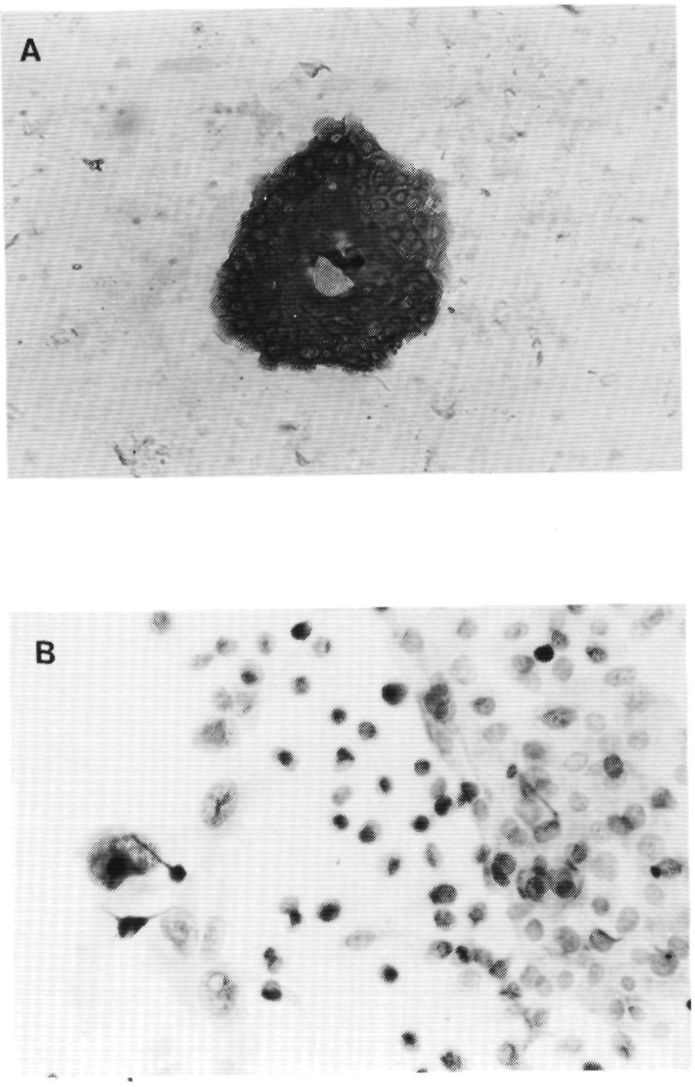


Figure 5 RCK102 (A) and anti-vimentin (B) staining of human cultured keratinocytes.

vious single parameter studies[23], indicating that we are not introducing artifacts by measuring more parameters at once.

Cell suspensions derived from psoriatic skin also could be subdivided into distinct subpopulations according to RKSE60 binding. In the keratin 10-posi-

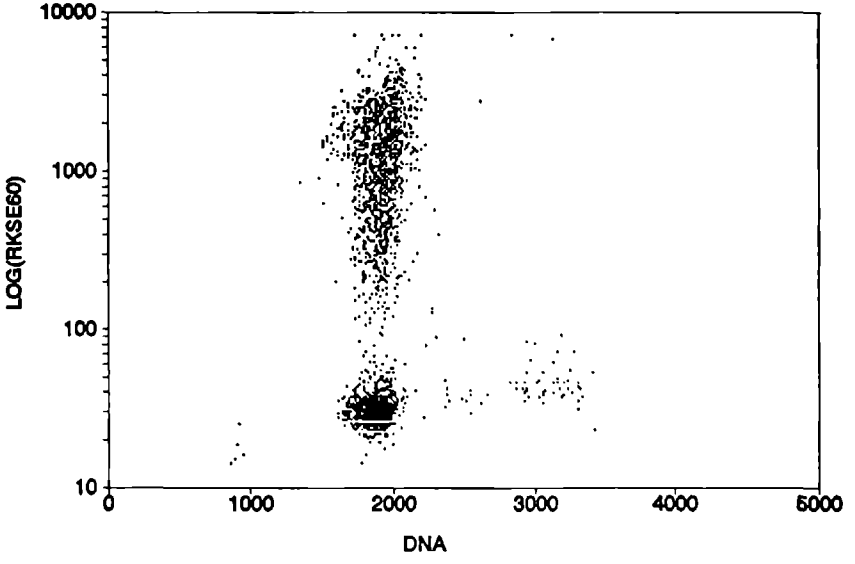


Figure 6 Typical two-parameter dot-plot, relative DNA content on the x-axis and keratin 10 antibody binding on the y-axis. The cells were derived from normal skin.

tive cell population proliferative activity is substantially lower, most cells having diploid DNA content. This is compatible with the suprabasal expression of keratin 10 and the supposition that suprabasal cells are differentiated and no longer capable of dividing.

Using the same staining protocol for Pab601 as for

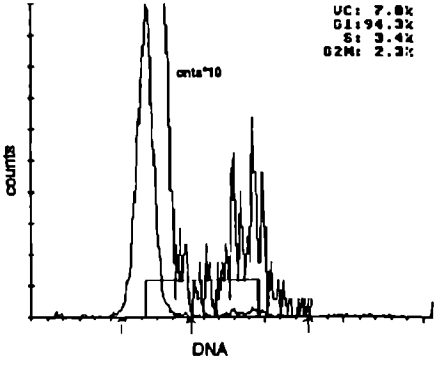


Figure 7 This figure shows a conversion of the data from figure 6 resulting in a DNA histogram.

RKSE60 no specific immunofluorescence could be observed by fluorescence microscopy, and an additional post-fixation step with acetone proved to be necessary. The fact that straight 70% ethanol fixation was not sufficient to allow the MAb Pab601 to bind to epidermal cells is presumably due to masking of the epitope. Using the extra fixation step, a reasonable separation between Pab601-positive and Pab601-negative cell populations could be shown in cell suspensions obtained from normal skin. Yet, in the case of cell suspensions derived from psoriatic skin, no separation between Pab601-positive and Pab601-negative cells could be obtained in the majority of the samples. About 40% of the keratinocytes from normal epidermis were Pab601-positive, including the majority of cells in S-phase and G_2M-phase of the cell cycle. Pab601-positive cells measured flow cytometrically are probably basal cells, since the sum of the numbers of RKSE60-positive, Pab601-positive and MVI-positive cells in cell suspensions derived from normal cells was 100% and the fact that the majority of cells in S-phase and G_2M-phase of the cell cycle stained with Pab601.

The appearance of keratin 16 in the lesional epidermis of psoriatic patients could be confirmed using FCM. K_s8.12-positive and K_s8.12-negative cell populations were clearly separated. In normal skin K_s8.12-positive cells were seen sporadically with a fluorescence intensity of the same order as seen in cells derived from psoriatic lesions. As the analysis was performed on a "per cell basis" cell populations expressing keratin 16 could be quantified accurately. A large variation between individual lesions with respect to numbers of keratin 16-positive cells was shown. When a standardized trauma such as tape-stripping was used, the variation between percentages of K_s8.12-positive cells appearing 16 h after stripping was much smaller. The variation in psoriatic lesional epidermis can be explained by variation between sites within the same plaque rather than inter-individual variation. As MAb analysis was done concomitantly with measurement of relative DNA content, at a functional level, binding of K_s8.12 was predominantly found to be restricted to diploid cells. This is in line with the suprabasal location found immunohistochemically. The clear separation shown by flow cytometric analysis is also in line with the discrete boundary seen in the frozen sections stained with K_s8.12.

In normal epidermis the vimentin-positive cell population will consist almost

exclusively of dendritic cells (melanocytes, Langerhans cells), in psoriatic lesional skin inflammatory infiltrate cells will be present in addition. Especially in the active phases of the psoriatic lesion, infiltrate cells, including monocytes, lymphocytes, and polymorphonuclear leukocytes, invade the epidermis[24]. However, the increased epidermal volume, and hence the increased number of keratinocytes under a certain surface[25], together with a reduction of the number of Langerhans cells[26], might account for the relative reduction of vimentin-positive cells in the psoriatic lesion. Using the method described in this study, the changes in the number of vimentin-positive cells in diseased epidermis can influence results obtained with antibodies directed against a subpopulation of keratinocytes. Two-color immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated antibodies could be used to express for instance the number of RKSE60-positive cells (PE-labelled) as a percentage of total epidermal keratinocytes (polyclonal anti-keratin antibody, FITC-labelled). In this way, interference of non-keratinocytes can be avoided. Using a single laser system, PI should be replaced by 7-amino-actinomycin D to allow simultaneous cell cycle analysis[27].

Figure 8 shows examples of Pab601 binding in keratinocytes from normal epidermis (8A), RKSE60 binding in keratinocytes from psoriatic lesion (8B), and K_s8.12 binding in keratinocytes from psoriatic lesion (8C), illustrating the association with cells having more than diploid DNA content (8A) and cells having diploid DNA content (8B and 8C), respectively.

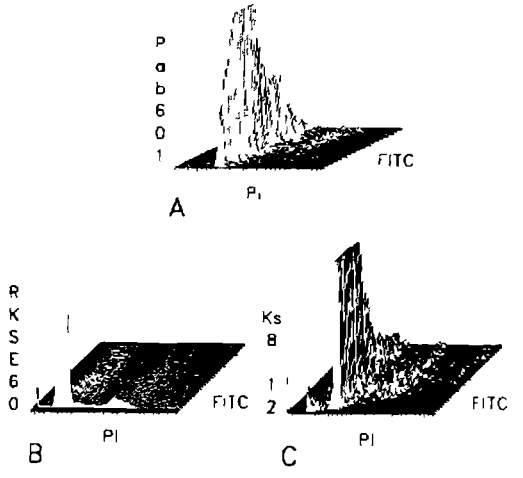


Figure 8 Two-parameter frequency histograms of antibody binding (FITC, y-axis) and relative DNA content (PI, x-axis), simultaneously A Pab601, B RKSE60, C. K_s8.12

We demonstrate here that flow cytometric quan-

tification of intermediate filament-type proteins in addition to relative DNA content provides a valuable approach for studies on epidermal pathology. Although in principle very simple, this approach is not very well documented in the literature. A recent review on the technique illustrates this very clearly[28]. In almost all studies until now, precipitation fixatives, such as ethanol and methanol have been used. One important reason to fix the cells is that primary and secondary antibodies must penetrate the plasma membrane and that the DNA must be susceptible for staining with the polar intercalating dye PI. Furthermore, fixed cells can be stored for periods up to a year at -20°C. Other approaches to introduce immunoglobulins into the cytoplasm are the use of detergent as demonstrated for anti-keratin antibodies by Staquet et al[29] or the use of lysolecithin with viable cells[30].

In summary this chapter illustrates that multiparameter characterization of epidermal subpopulations using flow cytometry is a useful novel tool for studies on skin pathology. Epidermal characteristics are assessed on a per cell basis and analyzed rapidly (>500 cells per second). The method is highly reproducible and since many cells are measured per sample statistical accuracy is high. When applied to cell suspensions derived from skin similar results were obtained compared to previous microscopic evaluation Additionally, we were able to show the potential value of MAb K_s8.12 as a new marker for hyperproliferation. Multiparameter FCM permits quantitative population analysis, extending our knowledge on topographical relations in the epidermis as visualized under the microscope.

4. References

1. Eichner R, Bonitz P, Sun T-T· Classification of epidermal keratins according to their immunoreactivity, isoelectric point, and mode of expression J Cell Biol 98 1388-1396, 1984

2. Fuchs E, Green H Changes in keratin gene expression during terminal differentiation of the keratinocyte Cell 19 1033-1042, 1980

3. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R The catalog of human cytokeratin polypeptides patterns of expression of specific cytokeratins in normal epithelia, tumors and cultured cells Cell 31·11-24, 1982

4 Woodcock-Mitchell J, Eichner R, Nelson WG, Sun T-T Immunolocalization of keratin polypeptides in human epidermis using monoclonal antibodies J Cell Biol 95 580-588, 1982

5. Fuchs E, Green H: The expression of keratin genes in epidermis and cultured epidermal cells. Cell 15:887-897, 1978

6. Kopan R, Traska G, Fuchs E: Retinoids as important regulators of terminal differentiation: examining keratin expression in individual epidermal cells at various stages of keratinization. J Cell Biol 105:427-440, 1987

7. Van Muljen GNP, Warnaar SO, Ponec M: Differentiation-related changes of cytokeratin expression in cultured keratinocytes and in fetal, newborn, and adult epidermis. Exp Cell Res 171:331-345, 1987

8. McGuire J, Osber M, Lightfoot L: Two keratins MW 50,000 and 56,000 are synthesized by psoriatic epidermis. Brit J Dermatol 111 (Supplement 2):27-37, 1984

9. Moll R, Moll I, Franke WW: Differences of expression of cytokeratin polypeptides in various epithelial skin tumors. Arch Dermatol Res 276:349-363, 1984

10. Weiss RA, Eichner R, Sun T-T: Monoclonal antibody analysis of keratin expression in epidermal diseases: a 48- and 56-kdalton keratin as molecular markers for hyperproliferative keratinocytes. J Cell Biol 98:1397-1406, 1984

11. De Waal RMW, Semeijn JT, Cornelissen IMH, Ramaekers FCS: Epidermal Langerhans cells contain intermediate-sized filaments of the vimentin type: an immunocytologic study. J Invest Dermatol 82:602-604, 1984

12. Murphy GF, Flynn TC, Rice RH, Pinkus GS: Involucrin expression in normal and neoplastic human skin: a marker for keratinocyte differentiation. J Invest Dermatol 82:453-457, 1984

13. Kanitakis J, Ramirez-Bosca A, Reano A, Viac J, Roche P, Thivolet J: Filaggrin expression in normal and pathological skin - A marker of keratinocyte differentiation. Virch Arch A Path Anat Histopat 412:375-382, 1988

14. Bauer FW, Boezeman JBM, van Engelen L, de Grood RM, Ramaekers FCS: Monoclonal antibodies for epidermal population analysis. J Invest Dermatol 87:72-75, 1986

15. Huszar M, Gigi-Leitner O, Moll R, Franke WW, Geiger B: Monoclonal antibodies to various acidic (type I) cytokeratins of stratified epithelia. Selective markers for stratification and squamous cell carcinomas. Differentiation 31:141-153, 1986

16. Ramaekers FCS, Puts JJG, Moesker O, Kant A, Huysmans A, Haag D, Jap PHK, Herman CJ, Vooijs GP: Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumors: an overview. Histochem J 15:691-713, 1983

17. Bauer FW, Crombag NHCMN, de Grood RM, de Jongh GJ: Flow cytometry as a tool for the study of cell kinetics in epidermis. Investigations on normal epidermis. Brit J Dermatol 102:629-639, 1980

18. De Mare S, van Erp PEJ, Ramaekers FCS, van de Kerkhof PCM: Flow cytometric quantification of human epidermal cells expressing keratin 16 in vivo after standardized trauma. Arch Dermatol Res 282:126-130, 1990

19. Bauer FW, Boezeman JBM: Flow cytometric methods in human skin with respect to cell cycle kinetics. in: Psoriasis: cell proliferation. Eds. Wright NA, Camplejohn RS. Edinburgh, Churchill Livingstone, pp 104-116, 1983

20. Rheinwald JG, Green H: Serial cultivation of strains of human keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331-344, 1975

21. Lane EB, Bartek J, Purkis PE, Leigh IM: Keratin antigens in differentiating skin. Ann N Y Acad Sci 455:241-258, 1985

22. De Mare S, van Erp PEJ, van de Kerkhof PCM: Epidermal hyperproliferation assessed by the monoclonal antibody K_s8.12 on frozen sections. J Invest Dermatol 92:130-131, 1989

23. Bauer FW, Crombag NHCMN, Boezeman JBM, de Grood RM: Flow cytometry as a tool for the study of cell kinetics in skin 2. cell kinetic data in psoriasis. Brit J Dermatol 104:271-276, 1981

24. Stadler R, Schaumberg-Lever G, Orfanos CE: Histology. In: Textbook of psoriasis. Eds. Mier PD, van de Kerkhof PCM. Edinburgh, Churchill Livingstone, pp 40-54, 1986

25. Bauer FW: Cell kinetics. In: Textbook of psoriasis. Eds. Mier PD, van de Kerkhof PCM. Edinburgh, Churchill Livingstone, pp 100-112, 1986

26. Mahrle G: Ultrastructure. in: Textbook of psoriasis. Eds. Mier PD, van de Kerkhof PCM. Edinburgh, Churchill Livingstone, pp 113-124, 1986

27. Rabinovitch PS, Torres RM, Engel D: Simultaneous cell cycle analysis and two-color surface immunofluorescence using 7-amino-actinomycin D and single laser excitation: applications to study of cell activation and the cell cycle of murine LY-1 B cells. J Immunol 136:2769-2775, 1986

28. Beck JLM, Vooijs GP, Ramaekers FCS: Application of antibodies in the flow cytometric analysis of benign and malignant cells. Sem Cancer Biol 1:181-188, 1990

29. Staquet MJ, Lawrence JJ, Albert J, Thivolet J: Flow cytometric sorting and keratin analysis of human epidermal basal cells. J Dermatol 14:1-8, 1987

30. Filippi JF, Tubiana N, Le Caër F, Sampol J, Carcassonne Y: Detection of intracytoplasmic immunoglobulins by flow cytometry. Nouv Rev Fr Hematol 28:297-302, 1986

CHAPTER III. CELL CYCLE KINETICS IN EPIDERMIS USING BrdUrd/IdUrd INCORPORATION

This chapter was based on the following publications:

Piet EJ van Erp, Paul PT Brons, Jan BM Boezeman, Gijs J de Jongh, Franz W Bauer, A rapid flow cytometric method for bivariate bromodeoxyuridine/DNA analysis using simultaneous proteolytic enzyme digestion and acid denaturation. Cytometry 9:627-630, 1988

Joris J Rijzewijk, Piet EJ van Erp, Jan BM Boezeman, Gijs J de Jongh, Paul D Mier, Franz W Bauer, The development of the bromodeoxyuridine technique for kinetic studies in human epidermis. Epithelia 1:323-333, 1989

CHAPTER III. CELL CYCLE KINETICS IN EPIDERMIS USING BrdUrd/-IdUrd INCORPORATION

1. Introduction 49
2. Methods & Materials 50
2.1. Cell Culture and Tissue Preparation (50)
2.2. BrdUrd and IdUrd Labelling (51)
2.3. Immunohistochemistry (51)
2.4. Denaturation and Staining for Flow Cytometry (51)
2.5. Flow Cytometry (52)
3. Results and Discussion 52
4. References 58

1. Introduction

Bromodeoxyuridine (BrdUrd) and iododeoxyuridine (IdUrd) are thymidine analogues which are incorporated into DNA synthesizing nuclei. These nuclei can be visualized by an immunofluorescence or an immunoperoxidase technique using monoclonal antibodies against BrdUrd[1]. This technique is very useful for kinetic studies For instance, it can be used as an alternative for [^{3}H]thymidine autoradiography and has certain advantages over it Firstly, there is no need for facilities required for work with radioactive tracers Secondly, the cells labelled with BrdUrd can be detected more quickly since the long exposure time needed for autoradiographs can be omitted and thirdly, BrdUrd and especially IdUrd may under some conditions be used *in vivo* even in human beings[2,3].

This technique has therefore found wide application in flow cytometry. For kinetic investigations a fluorochrome such as propidium iodide (PI) is used that stains DNA quantitatively, providing a measure of the DNA content per cell With the BrdUrd-technique information can be obtained on the number of synthesizing cells Simultaneous measurement of incorporated BrdUrd (or IdUrd) and relative DNA content by flow cytometry allows detailed analysis of cell cycle kinetics[2,4,5,6,7]. Several immunochemical procedures for cell staining have been described using anti-BrdUrd and PI, measuring simultaneously

DNA synthesis and relative DNA content[8,9,10,11]. Binding of the monoclonal anti-BrdUrd antibody requires regions of single-stranded DNA while PI is an intercalating dye which therefore requires double-stranded DNA. Acid treatment[9] or thermal denaturation[10] have been used most often, giving the best compromise. Recently, improvements of these techniques have been published using additional cell treatment, such as extraction of chromatin proteins by detergent, reduction of autofluorescence by sodium borohydride[8] or an extra protein degradation step using a proteolytic enzyme[11]. However, these protocols are either time consuming or at least in our hands cause substantial cell loss, especially when applied to haematopoietic cells.

This chapter describes the investigations on the influence of the variables involved in the BrdUrd/IdUrd staining protocol, such as method of fixation, DNA hydrolysis and enzymatic degradation. As a result we have developed protocols for cryostat sections of skin, for keratinocytes cultured on coverslips and for epidermal cell suspensions obtained either directly from human skin or from cultured keratinocytes. Haematopoietic cells were used to show the general applicability of the flow cytometric technique.

2. Methods & Materials

2.1. Cell Culture and Tissue Preparation

Human neonatal foreskin keratinocytes were cultured on 3T3 feeder cells[12] as described in chapter II at p. 33. EDTA treated, trypsinized keratinocytes from the first 1-2 passages were used in the experiments.

Human skin biopsies, 3 mm in diameter and thickness about 0.3 mm, obtained from healthy volunteers, were cut as previously described[13]. Epidermal cell suspensions were prepared by trypsinization[14].

Human bone marrow was collected in sterile buffered acid-citrate dextrose (ACD, formula A, pH = 7.0). Erythrocytes were removed by gradient centrifugation at 500 g for 30 minutes on 1.077 kg/l Ficoll-Isopaque (Pharmacia, Uppsala, Sweden). The nucleated cells from the interphase were washed twice in phosphate-buffered saline (PBS) and pre-incubated in RPMI 1640 (Boehringer Mannheim, FRG), supplemented with 100 mg/ml streptomycin, 2.10^{-3} M glutamine and 10% v/v fetal calf serum (Flow Laboratories, Irvine, Scotland).

2.2. BrdUrd and IdUrd Labelling

Cultured cells and human bone marrow cells (10^6 cells/ml) were pulse-labelled *in vitro* (15, 30 or 60 min) in medium containing BrdUrd (Serva, Heidelberg, FRG) or IdUrd (Sigma, St.Louis, USA). The final BrdUrd and IdUrd concentration was 10 μM. Cells were harvested, washed in ice-cold PBS fixed in ice-cold 70% ethanol and stored at -20°C until use. Skin biopsies were floated, dermal face downward, on 2 ml medium 199 containing 135 μg/ml BrdUrd or IdUrd (final concentration) at 37°C. After 1 h the tissue was trypsinized, and isolated cells were fixed in ice-cold 70% ethanol and stored at -20°C until use.

2.3. Immunohistochemistry

For the preparation of frozen sections, skin biopsies were washed in saline and embedded in Tissue-Tek OCT compound (Miles, Naperville, USA) in the cryostat at -20°C. Sections of 8μm were cut and air dried. The hydrolysis steps are described in the Results & Discussion section at p. 54. On both cryostat sections and coverslips an indirect immunoperoxidase staining was performed, using DAKO-BrdUrd (Dakopatts, Copenhagen, Denmark) as the primary antibody, peroxidase-conjugated rabbit anti-mouse IgG (RAMPO) (Dakopatts, Copenhagen, Denmark) as the second step, and amino ethylcarbazole (AEC) (Sigma, St.Louis, USA) together with H_2O_2 as enzyme substrates. Details are described in chapter II at p. 34.

2.4. Denaturation and Staining for Flow Cytometry

Between 0.5-1.0x10^6 ethanol-fixed cells were used for further preparation. Cells were washed once with PBS. Acid hydrolysis was tested in a variety of conditions with respect to pH, time and temperature. The effect of pepsin (Serva, Heidelberg, FRG) was investigated; experimental conditions are specified in the Results and Discussion section at p. 55. Hydrolysis was terminated with excess 0.1 M $Na_2B_4O_7$. After a minimum of 2 washes with PBS, denatured and/or protein-digested cells were pelleted. Cells were resuspended in 50 μl PBS containing 0.5% v/v Tween 20 (Sigma, St. Louis, MO, USA) and 5 μl FITC-conjugated anti-BrdUrd (Becton & Dickinson, Mountain View, CA, USA) was added. After 30 min incubation at room temperature in the dark, 500 μl PBS was added, cells were sedimented and washed once with PBS. Two other primary antibodies directed against BrdUrd, DAKO-BrdUrd (Dakopatts,

Copenhagen, Denmark) and HN-IU (a generous gift from the department of Haematology, University of Nijmegen), were used in addition. A second incubation-step with RAMFITC (Dakopatts, Copenhagen, Denmark) was necessary to visualize these antibodies. The cells were washed and resuspended in 400 μl PBS containing 20 μg/ml PI (Calbiochem, San Diego, CA, USA) and incubated for 15 min with 50 μl 1% w/v RNAse (Sigma, St. Louis, MO, USA). Prior to flow cytometric analysis cell suspensions were filtered through gauze (mesh 50 μm, van Wijk, Santpoort, The Netherlands).

2.5. Flow Cytometry

Cells stained with FITC and PI were measured with a flow cytometer 50H (Ortho Instruments, Westwood, CA, USA). The 488 nm line of a 5 W Argon ion laser was used for excitation. A 580 nm dichroic mirror, a high-pass filter RG630 nm for red, and a band-pass filter 515/530 nm for green were used. If possible, 5×10^4 cells were measured at a flow rate of approximately 100 cells per second. The area of the green fluorescence signal and both area and peak value of the red fluorescence signal were recorded in list mode. The ratio area:peak discriminates between artifacts due to doublets of diploid cells and real single tetraploid (or late S) cells, when intact cells are used[15].

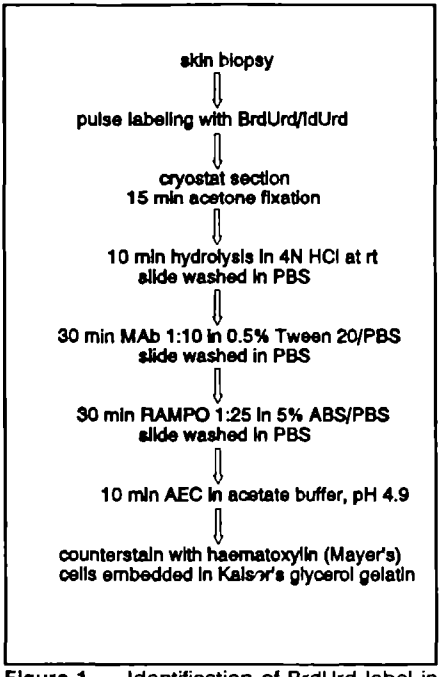


Figure 1 Identification of BrdUrd label in skin cryostat sections.

3. Results and Discussion

The incorporated BrdUrd (or IdUrd) is detected immunocytochemically in partially denatured DNA using a MAb[1]. The applicability depends on selected conditions to allow antibody binding. The integrity and architecture of tissue and cells should be preserved as far as possible for additional morphological

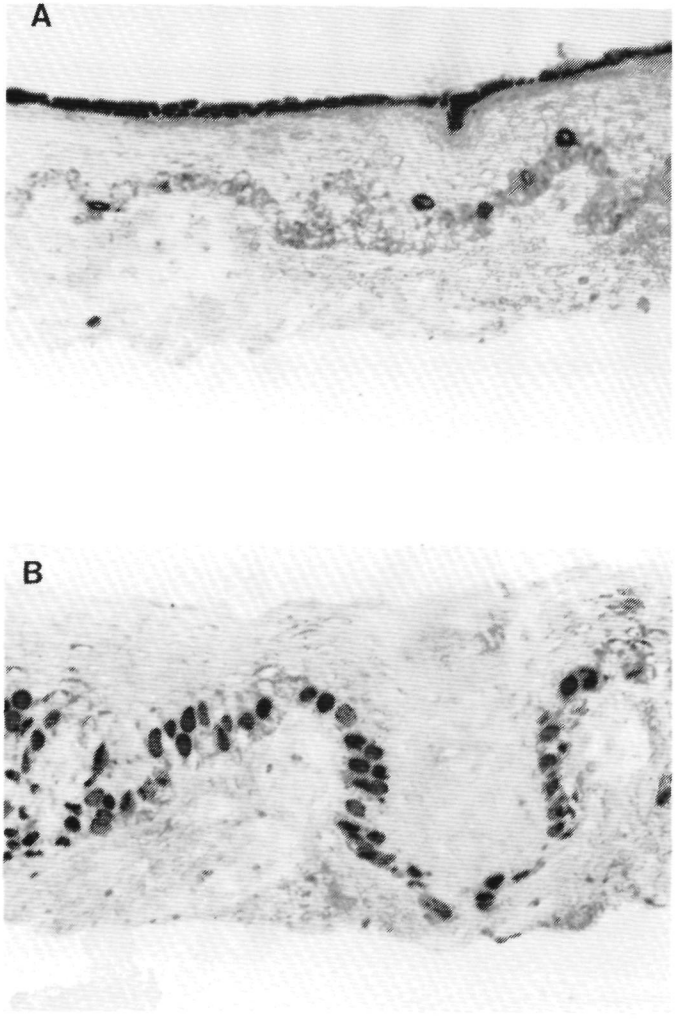


Figure 2 Immunoperoxidase staining on cryostat sections of S-phase cells after BrdUrd incorporation. A: normal human skin. B: epidermis 40h after sellotape stripping.

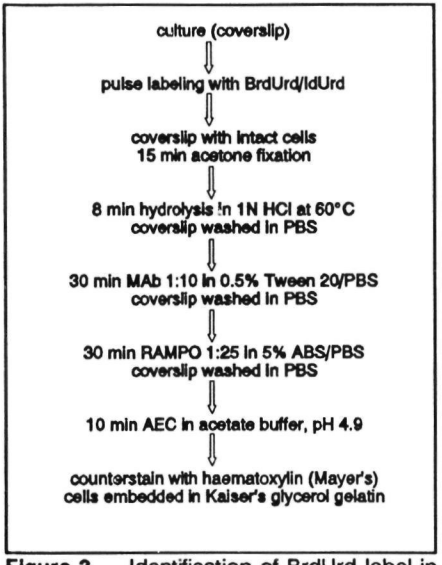


Figure 3 Identification of BrdUrd label in intact human keratinocytes cultured on coverslips.

and immunocytochemical characterization. Another requirement in the case of flow cytometric analysis is the appropriate staining of DNA with PI. BrdUrd (or IdUrd)-staining requires regions of single-stranded DNA while PI is an intercalating dye requiring double-stranded DNA.

The final protocol for the staining of skin sections is shown in figure 1. Acetone fixation was essential to obtain satisfactory results. Fixation with 70% ethanol did not work. Acid hydrolysis was tested at different HCl strengths in the range 0.1 N to 4 N HCl. Best results in terms of immunostaining and of preservation of histological details were obtained using 4 N HCl for 10 min at room temperature. The appearance of sections stained using this technique is illustrated in figure 2.

The protocol for *in situ* immunostaining of cultured keratinocytes is described in figure 3. Culture on coverslips made it easier to study variables. Moreover, acetone fixation proved to be necessary, which causes problems in many plastic culture dishes. Alkaline denaturation with 0.08 N NaOH, according to a manufacturer's protocol (Becton & Dickinson), was deleterious. After a short period the cultured cells became slimy and detached from the plastic. Acid hydrolysis, as

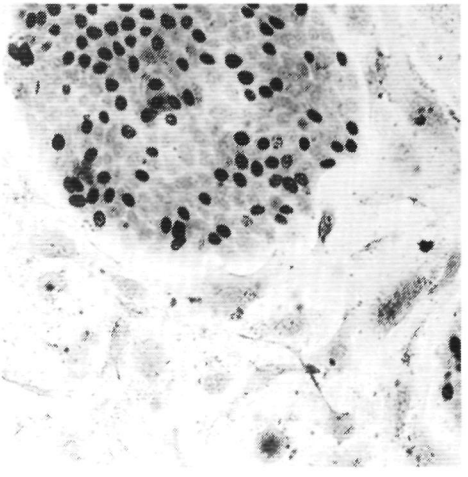


Figure 4 Immunoperoxidase staining of cells in S-phase of the cell cycle, after BrdUrd/IdUrd incorporation in a growing keratinocyte colony *in vitro*.

used before, i.e. 4 N HCl for 10 min at room temperature was also unsuccessful. New conditions for acid hydrolysis[16], namely 8 min hydrolysis in 1 N HCl at 60°C gave positive results. Typical results are shown in figure 4; it is seen that the architecture of the cell colonies and also cellular details are well preserved with this technique.

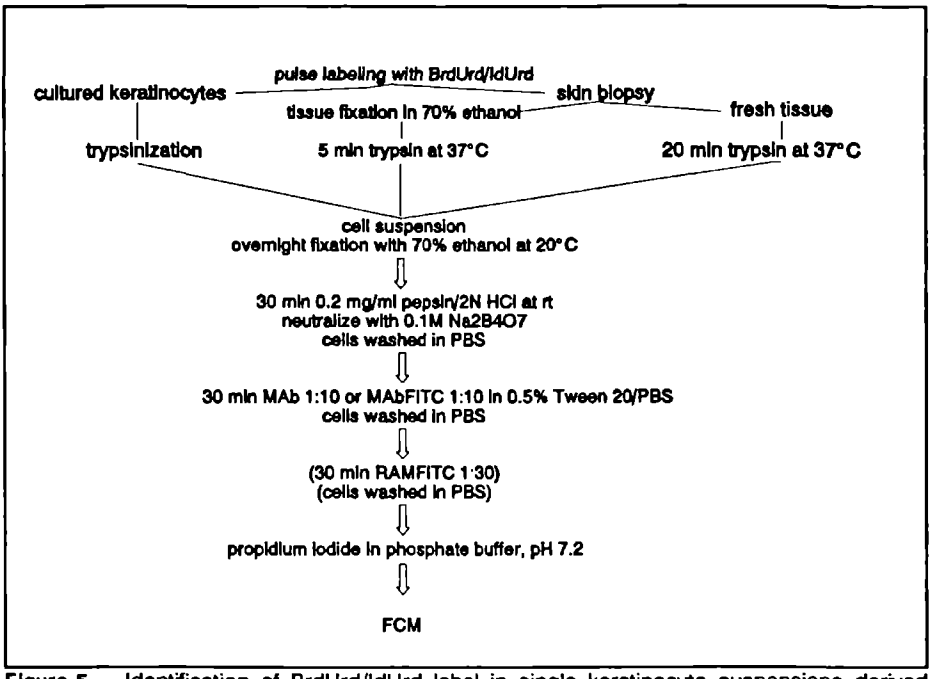


Figure 5 Identification of BrdUrd/IdUrd label in single keratinocyte suspensions derived from skin biopsies and cultures.

The final protocol for immunostaining of cell suspensions is shown in figure 5. Here it proved to be irrelevant from which source they were derived, either from cultured keratinocytes or from biopsies. Once fixed in cold 70% ethanol the same procedure was employed. It is important that it appears possible to fix the specimens in cold ethanol immediately after the biopsy is taken.

Acid hydrolysis at different HCl strengths (range 0.1 N-4 N), and different incubation temperatures (room temperature, 37°C, 60°C) are generally used[2,4,18]. However, with cultured keratinocytes or cells derived from human skin biopsies pulse-labelled for 1 h with 10 μM or 135 μg/ml BrdUrd, respectively, we were not able to obtain the results reported with these staining protocols (figure 6B). Concentrations greater than 2 N HCl and/or longer incu-

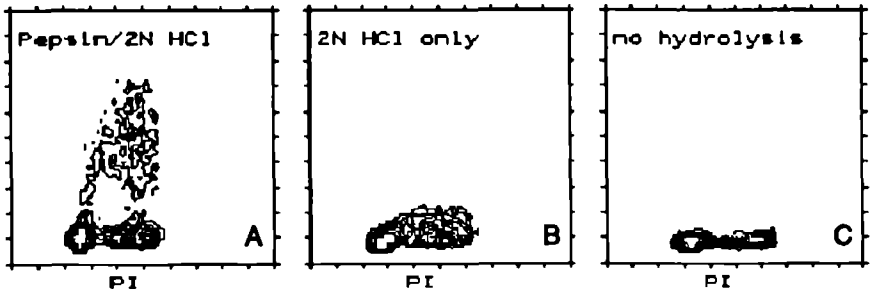


Figure 6 Bivariate BrdUrd/DNA analysis of cultured human keratinocytes. Comparison of pretreatment with 0.2 mg/ml pepsin in 2N HCl (A), 2N HCl only (B), and without treatment (C).

bation times lead to increased cell loss, naked nuclei or even total destruction of the nuclei and much debris. Preincubation of ethanol-fixed cells with 0.4 mg/ml pepsin in 0.1 N HCl for 30 min at room temperature followed by denaturation using 2 N HCl[11] resulted in some improvement. As expected, this procedure resulted in naked nuclei, but flow cytometric analysis was hampered because of large amounts of debris. Substantial improvement was obtained with simultaneous acidic denaturation and pepsin digestion showing high sensitivity (figure 6A) and low background fluorescence (figure 6C). Pepsin has a pH optimum of 1.5 but is still sufficiently active in 2 N HCl. We tested pepsin concentrations from 0.1 mg/ml to 1.0 mg/ml in 2 N HCl at room temperature; 0.2-0.4 mg/ml giving optimal results. Surprisingly, 0.2 mg/ml pepsin in 2 N HCl kept most of the cytoplasmatic structure intact as visible with a phase-contrast light microscope. However, when the neutralization step with $Na_2B_4O_7$ was omitted and the cells were washed with PBS alone, only naked nuclei were obtained. Comparison of DNA histograms obtained before and after hydrolysis showed that the combined pepsin/denaturation step did not affect the distribution of cells over the different cell cycle phases, as detected with PI. Moreover, this method resulted in DNA histograms with remarkably lower CVs for the staining with PI compared to acid denaturation only, although the absolute intensity of the red signal was somewhat decreased. In terms of sensitivity, the procedure described above is better or at least comparable with results obtained with other protocols[8,11]. The method described allows detection of S-phase cells after a labelling period with

BrdUrd as short as 5-10 min. Even when only 0.5-1.5% of all cells were in S-phase as is the case in normal human epidermis, BrdUrd-positive cells were clearly separated from G_0G_1 and G_2M cells after a 15 min pulse. Cell recoveries were greater than 85% which can be attributed to the relative mildness of this procedure and/or to the reduction of the number of incubation and wash steps; the total processing including measurement, when using a direct immunofluorescent technique, can be completed in less than 2 h.

Figure 7A and 7B illustrate that this procedure can be applied to cell types other than keratinocytes. Human bone marrow cells were pulse-labelled with 10 μM BrdUrd for 60 min, and after overnight fixation in 70% ice-cold ethanol, the cells were stained for BrdUrd incorporation and relative DNA content as described for epidermal cells. Similar bivariate BrdUrd/DNA distributions were obtained compared to stained epidermal cells and the small CVs of the DNA histograms (< 1.5%) were striking.

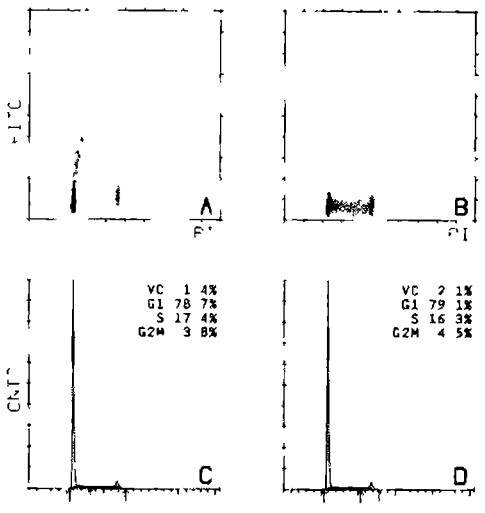


Figure 7 Bivariate BrdUrd/DNA analysis of human bone marrow cells pulse-labeled *in vitro*. A: treatment with pepsin/HCl, B: without hydrolysis.

In conclusion, the immunohistochemical protocols described are relatively simple, and maintain reasonable morphological integrity of the cells. This is also true for the novel flow cytometric procedure, which in addition is applicable to a variety of cells, including cultured keratinocytes, cells derived from solid tissue, and human bone marrow cells.

4. References

1. Gratzner HG: Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. Science 218:474-475, 1982

2. Wilson GD, McNally NJ, Dunphy E, Karcher H, Pfragner R: The labelling index of human and mouse tumours assessed by bromodeoxyuridine staining in vitro and in vivo and flow cytometry. Cytometry 6:641-647, 1985

3. Brons PPT, van Erp PEJ, Pennings AHM, Boezeman JBM: Cell kinetics in acute myeloid leukemia and other hematological malignancies studied with in vivo iododeoxyuridine administration and flow cytometry. in: New Trends in Acute Leukemia. PAOG University of Nijmegen, Nijmegen, 1990, pp 49-58

4. Begg AC, McNally NJ, Shrieve DC, Karcher H: A method to measure the duration of DNA synthesis and the potential doubling time from a single sample. Cytometry 6:620-626, 1985

5. Dean PN, Dolbeare F, Gratzner H, Rice GC, Gray JW: Cell-cycle analysis using a monoclonal antibody to BrdUrd. Cell Tissue Kinet 17:427-436, 1984

6. Pallavicini MG, Summers LJ, Dolbeare FD, Gray JW: Cytokinetic properties of asynchronous and cytosine arabinoside perturbed murine tumors measured by simultaneous bromodeoxyuridine/DNA analysis. Cytometry 6:602-610, 1985

7. Shrieve DC, Begg AC: Cell cycle kinetics of aerated, hypoxic and re-aerated cells in vitro using flow cytometric determination of cellular DNA and incorporated bromodeoxyuridine. Cell Tissue Kinet 18:641-651, 1985

8. Beisker W, Dolbeare F, Gray JW: An improved immunocytochemical procedure for high-sensitivity detection of incorporated bromodeoxyuridine. Cytometry 8:235-239, 1987

9. Dolbeare F, Gratzner H, Pallavicini MG, Gray JW: Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. Proc Nat Acad Sci USA 80:5573-5577, 1983

10. Dolbeare F, Beisker W, Pallavicini MG, Vanderlaan M, Gray JW: Cytochemistry for bromodeoxyuridine/DNA analysis: stoichiometry and sensitivity. Cytometry 6:521-530, 1985

11. Schutte B, Reynders MMJ, van Assche CLMVJ, Hupperets PSGJ, Bosman FT, Blijham GH: An improved method for the immunocytochemical detection of bromodeoxyuridine labelled nuclei using flow cytometry. Cytometry 8:372-376, 1987

12. Rheinwald JG, Green H: Serial cultivation of strains of human keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331-344, 1975

13. Bauer FW, de Grood RM: Improved technique for epidermal cell cycle analysis. Br J Dermatol 95:565-566, 1976

14. Bauer FW, Crombag NHCMN, de Grood RM, de Jongh GJ: Flow cytometry as a tool for the study of cell kinetics in epidermis. Investigations on normal epidermis. Br J Dermatol 102:629-639, 1980

15. Bauer FW, Boezeman JB: Flow cytometric methods in human skin with respect to cell cycle kinetics. in: Psoriasis: cell proliferation. Eds. Wright NA, Camplejohn RS. London, Churchill Livingstone, 1983, pp 104-116

16. Wynford-Thomas D, Williams ED: Use of bromodeoxyuridine for cell kinetic studies in intact animals. Cell Tissue Kinet 19:179-182, 1986

CHAPTER IV. EXPRESSION OF THE KI-67 ANTIGEN. ITS RELATION TO THE CYCLING CELL POPULATION IN HUMAN EPIDERMIS

Parts of this chapter were based on the following publications:

Joris J Rijzewijk, Piet EJ van Erp, Franz W Bauer, Two binding sites for Ki67 related to quiescent and cycling cells in human epidermis. Acta Derm Venereol (Stockh) 69:512-515, 1989

Piet EJ van Erp, Arie HM Pennings, Paul PT Brons, Gijs J de Jongh, Ki-67 measurements in human epidermis in vivo and cultured human keratinocytes: Optimization of cytochemical techniques. Manuscript in preparation.

CHAPTER IV. EXPRESSION OF THE KI-67 ANTIGEN. ITS RELATION TO THE CYCLING CELL POPULATION IN HUMAN EPIDERMIS

1. Introduction 63
2. Methods & Materials 64
2.1. Skin Sampling and Preparation of Cell Suspensions (64)
2.2. Human Keratinocyte Culturing (64)
2.3. Immunohistochemical Procedure (65)
2.4. Mitogen-Stimulated Lymphocyte Cultures (65)
2.5. Storage of Mononuclear Cell Suspensions (66)
2.6. Immunofluorescence Procedure and Staining Methods (66)
2.7. Flow Cytometry (67)
3. Results & Discussion 68
4. References 77

1. Introduction

Ki-67 is a monoclonal antibody (MAb) which recognizes a human nuclear proliferation-associated antigen. This antibody was described for the first time by Gerdes et al in 1983[1]. The antigen is expressed by cells in late G_1, S, G_2, and M-phase of the cell cycle, at least in the case of human peripheral blood lymphocytes[2]. The existence of such human nuclear antigens associated with proliferation has been suggested before. These antigens were detected by the use of autoantibodies found in sera of patients with lupus erythematosus (LE) and in sera of leukemic patients[3,4,5]. The antigen which binds Ki-67 has not been identified yet. Recently, another proliferation-associated antigen has been characterized, (PCNA)/cyclin. This antigen has been proven to be a 36kD intranuclear polypeptide, and is an auxiliary protein of the enzyme DNA polymerase δ[6]. It is this antigen to which the autoantibodies produced by LE patients are directed. Despite the fact that there are also MAb available directed against (PCNA)/cyclin[7], these antibodies are found to be less suitable for cell cycle analysis than Ki-67. The main reason for this is that cyclin is not present throughout the cell cycle of proliferating cells. A third antibody intro-

duced recently, is a MAb against DNA-methyltransferase[8,9]. However, this antibody is not commercially available, and is not yet fully characterized with respect to its expression during the cell cycle.

The reason for using Ki-67 and other antibodies recognizing proliferation-associated antigens is that until now there is no direct marker available which can discriminate between cycling cells and resting (G_0) cells. Boezeman et al[10], using a mathematical analysis of DNA distributions at different times following injury, have shown that a G_0 population does in fact exist in human epidermis *in vivo*; these authors demonstrated that under normal circumstances only a minority of cells are actively cycling.

In this chapter we describe studies to show that the Ki-67 antigen is present in normal and hyperproliferative epidermis *in vivo*, and in cultured human keratinocytes. Furthermore, we have investigated the possibility of measuring Ki-67 binding in keratinocytes flow cytometrically. Optimization of the flow cytometric technique was carried out with activated human peripheral blood lymphocytes.

2. Methods & Materials

2.1. Skin Sampling and Preparation of Cell Suspensions

Shave biopsies were taken as described in chapter II at p. 33 using a standard technique[11] from normal skin of healthy volunteers, from skin 40 h after tape-stripping, and from lesional skin of psoriatic patients. Biopsies were frozen directly in liquid nitrogen, and stored at -80°C until use. Cell suspensions were prepared from fresh tissue by trypsinization as described previously[12]. Cells were fixed according to the protocol described in the Results & Discussion section at p. 71.

2.2. Human Keratinocyte Culturing

Human neonatal foreskin keratinocytes were cultured on 3T3 feeder cells[13]. The detailed procedure is given in chapter II at p. 33. In some experiments keratinocytes were grown on thermonox acetone-resistant plastic coverslips. EDTA treated, trypsinized keratinocytes or coverslips with keratinocyte colonies from the first 1-2 passages were used in all experiments.

2.3. Immunohistochemical Procedure

Frozen biopsies were embedded in Tissue-Tek II O.C.T. compound (Miles Scientific, Naperville, USA) in a cryostat at -20°C. Sections of 8 μm were cut and fixed directly in acetone for 10-20 minutes. At a later stage fixation was carried out in acetone-ether (60/40 v/v) for 20 min. Fixed and air-dried sections were either kept at -80°C or used directly. The basic staining protocol is similar to the procedure for MAb against intermediate filament proteins described in chapter II at p. 34 of this thesis. Ki-67 concentration was tested in the range 1:4 to 1:50. Higher Ki-67 concentrations resulted in more nuclear signal. However, with dilutions in the range 1:4 to 1:10 the background signal (in particular cytoplasmic labelling of basal cells) interfered with the nuclear staining. Every batch of peroxidase-conjugated second antibody was tested for optimum concentration. Although small variations did exist, a dilution of 1:25 was generally used.

2.4. Mitogen-Stimulated Lymphocyte Cultures

Mononuclear cell suspensions were prepared from peripheral blood of volunteers using a 1.077 kg/l Ficoll-Isopaque (Pharmacia, Uppsala, Sweden). The cells were kept three days in RPMI 1640 (Boehringer, Mannheim, FRG), supplemented with 100 mg/ml streptomycin, 2 mM glutamine, and 10% v/v fetal calf serum (Flow Laboratories, Irvine, Scotland). Lymphocytes were stimulated in vitro using 60 μg/ml phytohemagglutinin (PHA) (Sigma, St.Louis, USA). A control culture without mitogen was grown as well. The effect of PHA stimulation was evaluated by continuous labelling with iododeoxyuridine (IdUrd) and flow cytometric DNA/IdUrd analysis. IdUrd was added to the cell suspensions at day 2 of stimulation with PHA. The final concentration was 10 μM. After an incubation period of 24 h cells were washed with PBS and fixed with ice-cold 70% ethanol. Samples were stained with anti-BrdUrd and RAMFITC using the pepsin/HCl protocol described in chapter III. All experiments were carried out in duplo and every time a blank was included (in duplo). PBS-washed 3ml samples contained $3x10^6$ cells. Each sample was divided into 6 portions. Centrifuge steps of unfixed cells were done at 1400 rpm for 5 min (4°C) and in the case of fixed cells 5 min 2000 rpm at 4°C was used.

2.5. Storage of Mononuclear Cell Suspensions

In order to evaluate the effect of storage, parallel samples were stored using different storage conditions for 1 week. The storage method used depended on the staining method to be used afterwards. One of 6 portions was frozen using a standard DMSO freezing protocol for vital cells. Two portions were stored at -80°C after a short fixation in paraformaldehyde at room temperature followed by methanol -20°C treatment. Penetration of the antibodies into cells fixed in suspension was improved by adding lysolecithin (L-α-lysophosphatidylcholine, Type I, Sigma, St.Louis, USA) to the paraformaldehyde solution[14]. One portion was fixed in acetone at -20°C and stored at -80°C. Finally, two portions were fixed in 70% ethanol at -20°C and stored at -80°C.

2.6. Immunofluorescence Procedure and Staining Methods

Freshly prepared cells or cells stored according to the procedures described above, were divided into 6 portions and stained as follows. Method 1: cells were fixed and labelled according to Palutke et al[15]. In brief, cells were

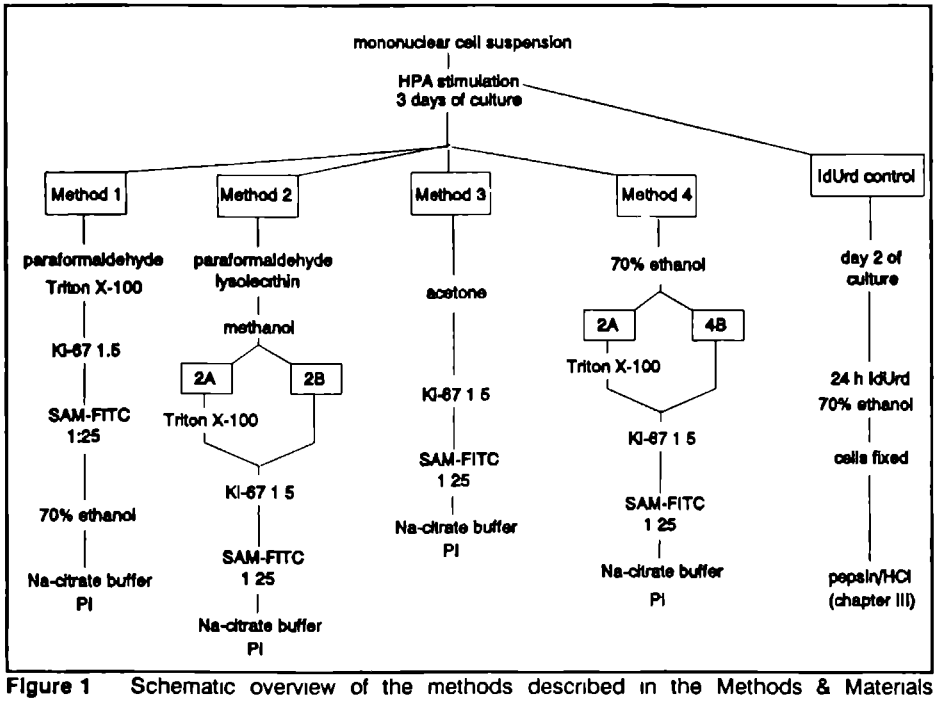


Figure 1 Schematic overview of the methods described in the Methods & Materials section for bivariate measurement of DNA/Ki-67

fixed in paraformaldehyde, followed by treatment with Triton X-100 to permit entrance of the antisera. Fixed cells were stained with Ki-67 (Dakopatts, Copenhagen, Denmark), diluted 1:5. FITC-conjugated swine anti-mouse immunoglobulins (SAMFITC, American Qualex International, La Miranda, USA) was used as the second antibody. From here, cells were treated in two ways. One sample was stained directly with PI in the presence of RNAse (see chapter II at p. 35), and one sample was stained with PI according to Brons et al[16]. The cells were spun down, the supernatant was discarded, and the pellet placed on ice for 10 min. Subsequently 900μl of an ice-cold hypotonic PI solution, containing 25 μg/ml PI, 0.1% w/v tri-sodium citrate dihydrate (Merck, Darmstadt, FRG), 0.01% RNAse, and 0.1% v/v Triton X-100, was added. Cells were kept overnight on ice. Method 2: cells were fixed with 1% paraformaldehyde containing 500 μg/ml lysolecithin for 2 min at room temperature[14]. After centrifugation the cells were resuspended in 2 ml methanol at -20°C for 10 min. The cells were then divided in two portions. Sample 2A was treated with 0.1% Triton X-100 in PBS and sample 2B was not. Antibody labellingconditions were as described in method 1. Method 3 was adapted from Sasaki et al[17]. The cells were fixed with acetone at -20°C for 30 min. After rinsing the cells in cold PBS thoroughly, the incubation steps with Ki-67 and SAMFITC were identical to method 1. Method 4: the fixative used in this method was ice-cold 70% ethanol. Fresh cells were fixed for a period of 20 min at -20°C. After this fixation period the cells were divided into two portions, as described in method 2. One sample was treated with 0.1% Triton X-100 (4A) and one sample was not (4B). Antibody labellingconditions and staining with PI were as in method 1. A schematic overview of the methods used is given in figure 1.

2.7. Flow Cytometry

Cell suspensions labelled with antibodies and stained with PI for relative DNA content were filtered through gauze (50 μm mesh) in order to remove clumps. From each sample 10^4 cells were measured in list mode. Area and peak signal of the red fluorescence from bound PI and the peak signal of the green FITC signal were measured using an Ortho 30H or an Ortho 50H flow cytometer (Ortho Instruments, Westwood, USA). Both flow cytometers were equipped as described in chapter II at p. 35.

3. Results & Discussion

Ki-67 binding was visualized on cryostat sections of normal skin, psoriatic skin, and skin 40 h after tape-stripping, using an indirect immunoperoxidase technique. Figure 2A illustrates a section of normal skin stained with Ki-67. Note that part of the epidermis, in particular the basal layer, shows cytoplasmic staining. In the case of hyperproliferative conditions, such as psoriasis (figure 2B) and skin 40 h after tape-stripping (figure 2C), a dramatic increase of nuclear Ki-67 staining was observed in the basal layers of the epidermis. In contrast, the cytoplasmic staining of the basal layer, as observed in normal epidermis, disappeared almost totally. Cultured human keratinocytes resemble in many ways hyperproliferative conditions *in vivo*.

Figure 3 shows that in a colony of exponentially growing keratinocytes the majority of the nuclei were stained with Ki-67 and that cytoplasmic staining was minimal.

Prior to the observations of Gelfant in 1976[18], it was generally believed that cell production was essentially regulated by variation of the cell cycle time (from 36 to >300 h) and that the majority of the germinative cells were actively cycling[19,20,21]. However, Gelfant found that a large proportion of the basal cells in normal human epidermis were not labelled with radioactive thymidine after an exposure time of 14 days. This was the first observation supporting the existence of a large G_0 cell population. More recently, other arguments in favour of the G_0 concept were presented in a flow cytometric analysis of proliferative activity following tape-stripping of epidermis[10,22]. The fact that cells are entering the S-phase synchronously following tape-stripping suggests that they were "resting" at a fixed position in the cell cycle. The validity of the hypothesis that, in analogy with other tissues, Ki-67 binds to the nuclei of actively cycling cells in epidermis is confirmed by the present observation of large numbers of Ki-67-positive nuclei in psoriatic epidermis (figure 2B), post-tape-stripping epidermis (figure 2C), and cultured human keratinocytes (figure 3), all hyperproliferative conditions with necessarily a large proportion of cycling cells. The present data indicate a growth fraction, i.e. the fraction of cycling cells in the germinative cell compartment, of about 5% and a G_0 cell pool of more than 90% for normal epidermis. As previously mentioned, in addition to the nuclear staining, cytoplasmic staining of the basal layer was frequently

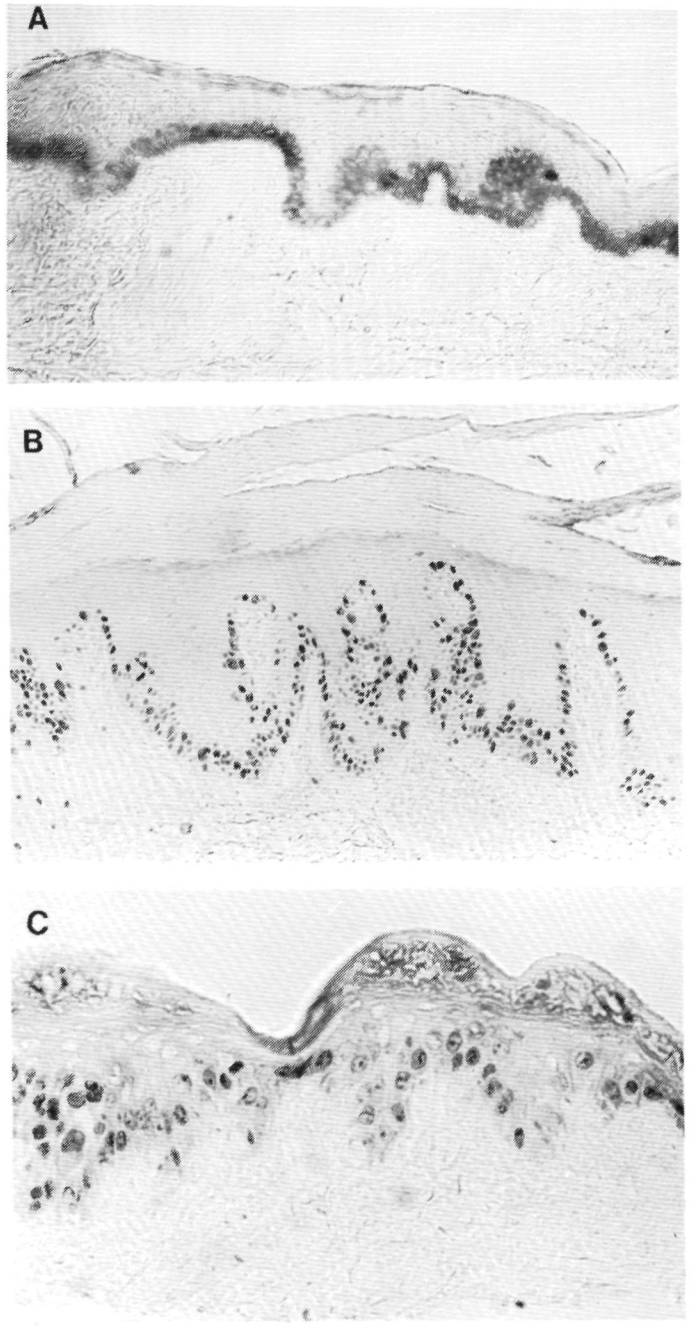


Figure 2 Examples of normal skin (A), psoriatic lesional skin (B), and skin 40 h after tape-stripping (C), staining with the proliferation-associated antibody Ki-67.

observed. It was dramatically reduced in the hyperproliferative psoriatic lesion (figure 2B), in tape-stripped epidermis (figure 2C), and in cultured keratinocytes (figure 3) compared with normal epidermis (figure 2A). Thus it seems that there is a negative relationship between cytoplasmic staining and proliferation. When germinative cells have entered the S-phase 40 h after tape-stripping, the numbers of Ki-67-positive nuclei are increased. Preceding this, there is a period (from 16 to 32 h) with only weak cytoplasmic and incidental nuclear staining. This interval might be related to the synchronized transition of numerous basal cells from the quiescent to the cycling state[22], the analogue of the specific Ki-67-negative G_1-phase between G_0 and S-phase, as has been suggested for lymphocytes[2] . It should be noted that the cytoplasmic and the nuclear binding sites are simultaneously present in the few cells in unstimulated epidermis that are cycling (figure 2A). This makes the possibility very unlikely of one binding site moving after stimulation from the cytoplasm to the nucleus. The nature of the nuclear binding site is not known, but is very probably a protein, since its expression in lymphocytes can be inhibited by cycloheximide[2]; the nature of the cytoplasmic binding site, however, remains obscure.

We have shown above, that the Ki-67 antigen is expressed in proliferating epidermal cells. Simultaneous staining of cells with Ki-67 and PI using FCM, would provide the opportunity to define more precisely the phenotype of proliferating cells. Furthermore, it would be possible to obtain a fast and statistically reliable statement on the proliferation of epidermal cells.

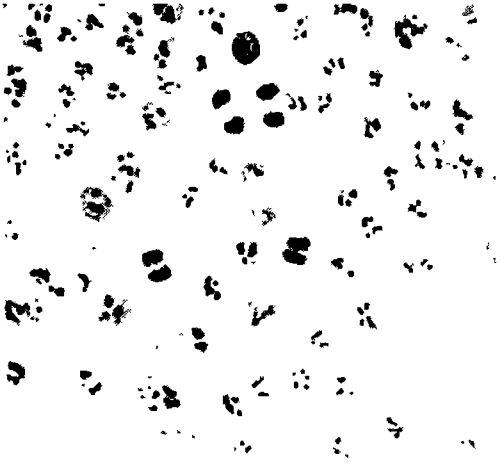


Figure 3 Ki-67 staining in a colony of exponentially growing human keratinocytes *in vitro*.

FCM is capable of measuring fluorescence of individual cells, therefore the peroxidase-conjugated second antibody used with the immunoperoxi-

dase technique on cryostat sections, had to be replaced by a FITC-conjugated antibody. The initial protocol used is shown in figure 4. The result was predictable. FCM cannot discriminate between the proliferation-associated nuclear staining and the cytoplasmic staining, especially when cells derived from normal epidermis were used.

To improve the flow cytometric staining method two alternative approaches were considered. Firstly, the most simple approach seemed to be separation of nucleus and cytoplasm by using detergents. Obviously, this would rule out the cytoplasmic staining totally. Although this technique has been applied to epidermal cells before[23,24], some critical remarks against the method can be made. The use of detergents will not only affect the lipids and proteins of the plasma membrane, but will also influence nuclear matrix proteins, including possibly the Ki-67 antigen The conditions to be chosen would be critical, and optimization of the technique would take much time. An even more important argument against the use of naked nuclei is the fact that simultaneous labelling with other keratinocyte-specific antibodies directed against cytoplasmic or membrane antigens becomes impossible. The epidermis is heterogeneous in its composition, and cell suspensions derived from a skin biopsy will contain many different cell types In order to differentiate between subpopulations additional antibodies will be necessary. For these reasons the use of naked nuclei was not our first choice

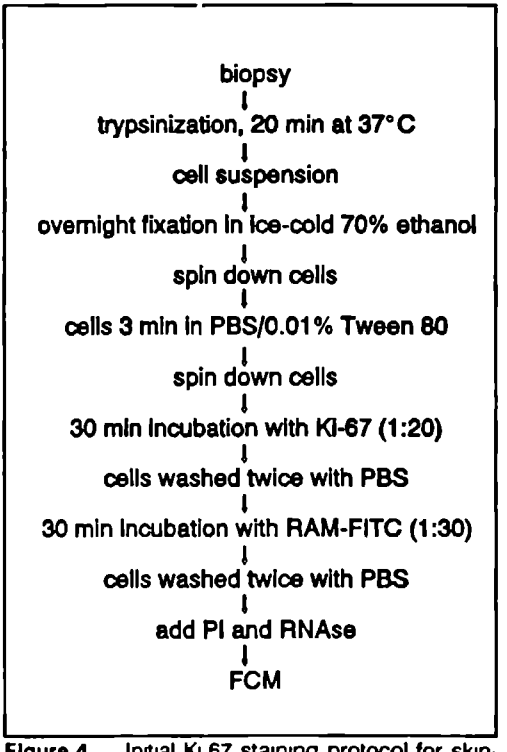


Figure 4 Initial Ki 67 staining protocol for skin-derived single cell suspensions

Our second approach was based on further optimization of existing methods None of the existing methods had been tested on epidermal cells

Cells of hematopoetic origin were used for testing the usefulness of Ki-67 as a proliferation marker in combination with FCM. For this reason we set up a study using lectin-stimulated peripheral blood lymphocytes. The aim of the study was, firstly, to evaluate the existing protocols for flow cytometric measurement of Ki-67 and relative DNA content. Secondly, a method described to analyze (PCNA)/cyclin staining was tested as Ki-67 staining method. Thirdly, we wanted to evaluate the effect of cell storage, because our group and also others had the experience that the Ki-67 antigen was not very stable when stored at -20°C after fixation in acetone or ethanol.

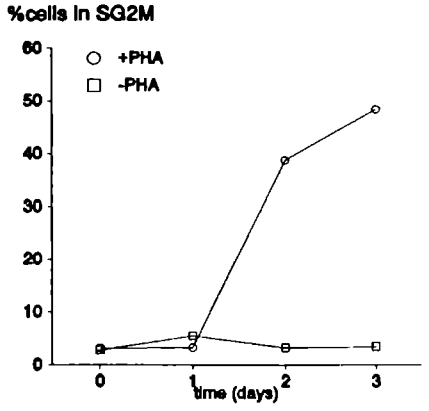


Figure 5 Percentage lymphocytes in S and G2M-phase after in vitro PHA stimulation

Mononuclear cell suspensions were prepared from peripheral blood as described in Methods and Materials at p. 65. The cells were stimulated *in vitro* using 60 μg/ml PHA. A control culture without mitogen was grown as well. The effect of PHA stimulation was evaluated by continuous IdUrd labelling and flow cytometric DNA/IdUrd analysis. Samples were stained with anti-BrdUrd and RAMFITC using the pepsin/HCl protocol described in chapter III. All experiments were carried out in duplo and every time a blank was included (in duplo). Figure 5 and 6 show

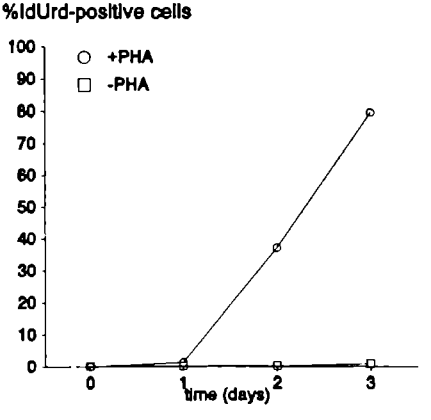


Figure 6 Percentage IdUrd-positive lymphocytes after stimulation in vitro with PHA.

the stimulating effect of PHA in time on percentage cells in S and G_2M-phase of the cell cycle, and percentage IdUrd-positive cells, respectively. At day 3 of stimulation more then 50% of the cells were in S and G_2M-phase of the cell cycle, and more then 80% of the cells were IdUrd-positive. Therefore, day 3 was taken as point of maximal stimulation.

The protocols tested are given schematically in figure 1, and details of materials used in Methods and Materials.

Table I Results obtained by different staining protocols for flow cytometric DNA/Ki-67 analysis. Unstimulated and PHA-stimulated lymphocytes were used as the source. The cells were not stored.

	unstimulated			PHA-stimulated		
method	CV	SG2M	Ki-67+	CV	SG2M	Ki-67+
1	5.4±1.7 (4)	1.6±0.4 (4)	0.9±0.2 (4)	4.0±0.8 (4)	21.2±1.9 (4)	70.6±4.7 (4)
2	9.8±4.4 (5)	2.0±0.2 (5)	2.4±0.5 (5)	11.5±0.5 (5)	22.1±0.9 (5)	61.6±5.4 (5)
3	34.5 (1)	10.9 (1)	23.2 (1)	7.4±3.1 (2)	25.4±1.0 (2)	71.8±0.7 (2)
4	3.9±0.3 (8)	2.3±0.2 (8)	0.6±0.1 (8)	4.6±0.3 (8)	24.7±0.5 (8)	72.5±0.7 (8)

Table I shows the results obtained by the 4 different staining protocols. Cells were fixed and stained directly without storage. Method 4 using 70% ethanol fixation gave the best results. The average absolute fluorescence of the Ki-67-positive population was lower compared to method 1 and method 3, but the blank value (incubations without Ki-67 added) and the fluorescent signal of the Ki-67-negative population were also very low. The second method, using paraformaldehyde together with lysolecithin in order to get optimal Ki-67 binding, was less successful. CVs were higher and the method was less reproducible. The method of Sasaki et al[17], using acetone fixation (method 3) resulted in our hands in moderate results. Although the absolute fluorescent signal was strong, the blank values were very high. Furthermore, CVs for the G_1-peak of the DNA histogram were much higher, comparing this method with the other staining protocols. The results obtained with the method

of Palutke et al[15] were much more satisfactory. There was a clear difference between Ki-67-positive and Ki-67-negative cells, and the CVs for the DNA histograms were low. This method, however, was more laborious compared to method 4. One other disadvantage was observed with this method. After storage of the paraformaldehyde-fixed cells at -80°C, the staining procedure resulted in marked cell loss.

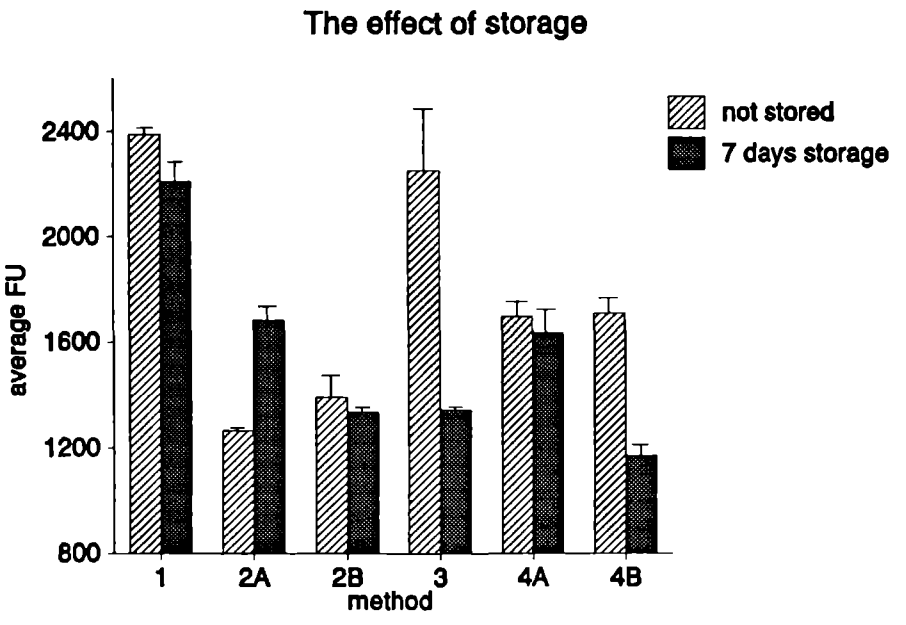


Figure 7 The effect of storage on HPA-stimulated lymphocytes using different staining protocols. On the y-axis the average fluorescent signal of the Ki-67-positive population is plotted.

The other effects of storage are summarized in figure 7. Method 2 and method 4 have been divided into A and B. In method 2A and method 4A an extra 10 min incubation step using 0.1% Triton X-100 was included before the Ki-67 incubation was started. Using this extra step, the average fluorescence of the Ki-67-positive population was of the same order as (4A) or even higher than (2A) that seen without storage (day 0). In contrast, 7 days storage of fixed lymphocytes and staining with Ki-67 without using Triton X-100 (method 2B, 3 and 4B) resulted in a significant reduction of the fluorescent signal. This result suggests that the use of precipitative fixatives such as methanol, ethanol, and acetone for longer periods can influence the penetration of Ki-67

and/or second antibody, resulting in lower fluorescent signals. Treatment of the cells with detergent seems to have a positive effect on the attainability of the Ki-67 antigen.

Comparing the four alternative staining methods, we found that the ethanol fixation method as used for structural cytoplasmic proteins (chapter II) and the BrdUrd/IdUrd staining technique (chapter III) gave the best results. When the fixed cells were stored for a longer period, an extra detergent step using Triton X-100 was essential. Figure 8 shows the difference between unstimulated and PHA-stimulated lymphocytes with respect to cells in S and G_2M-phase of the cell cycle, percentage IdUrd-positive cells, and percentage Ki-67-positive cells. Method 4 was used to stain the cells.

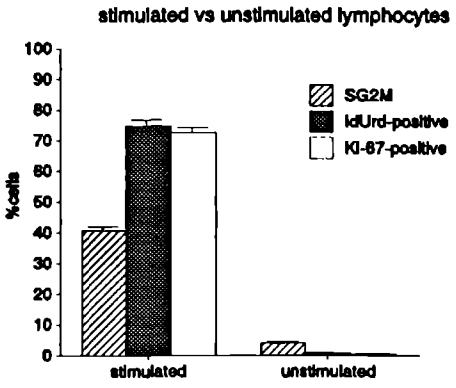


Figure 8 Comparison of proliferation markers using stimulated and unstimulated lymphocytes.

Finally, we have tested this staining method on a number of cell lines, including human cultured keratinocytes (NHK, figure 9). In an exponentially growing culture of keratinocytes more then 90% of the cells were Ki-67-positive. This result was confirmed by an immunoperoxidase staining on coverslips of a similar culture.

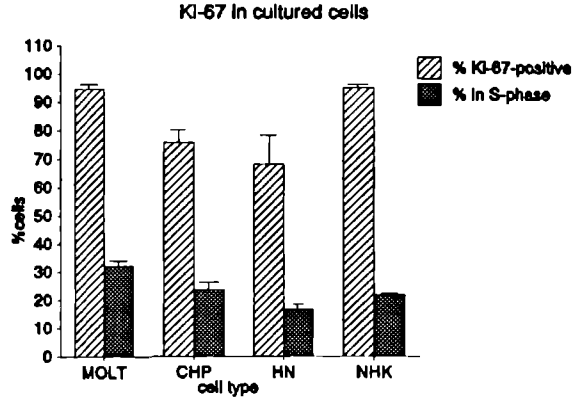


Figure 9 Ki-67 staining in a number of cell lines. The cultures were harvested when still in a subconfluent state and fixed immediately in ice-cold 70% ethanol.

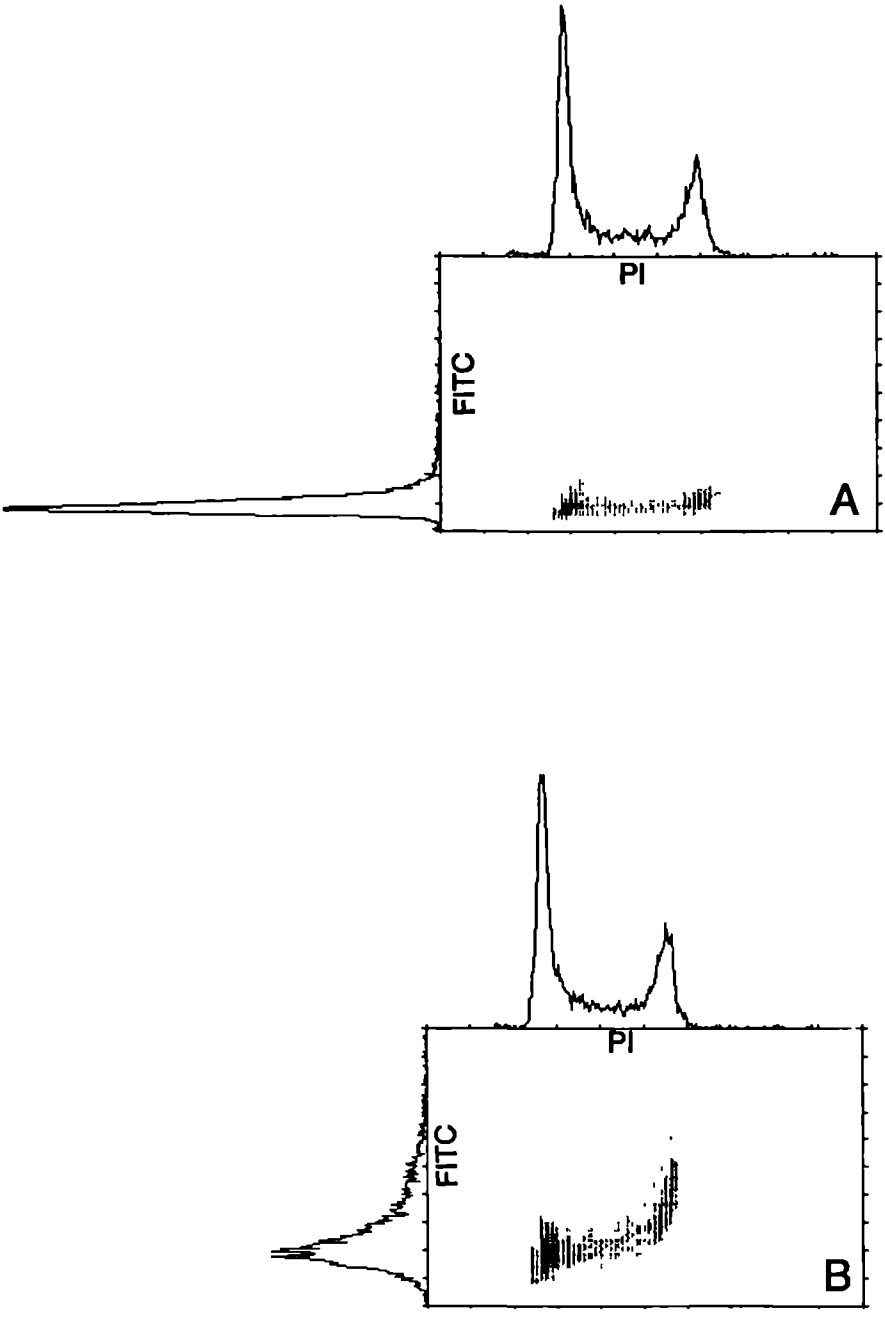


Figure 10 Ki-67/DNA scattergram obtained from exponentially growing keratinocytes (10B) The upper panel (10A) shows the negative control.

However, in contrast to human epidermal cells derived from a biopsy, cytoplasmic staining using Ki-67 was absent in cultured keratinocytes. This is in line with the results obtained with psoriatic or tape-stripped (hyperproliferative) epidermis, but also under confluent and low-serum conditions we could not demonstrate any significant cytoplasmic staining.

Figure 10B illustrates a typical two-parameter measurement of exponentially growing keratinocytes stained with Ki-67 and PI. Green fluorescence (Ki-67 binding) increases with cell cycle progression. RKSE60, a monoclonal antibody recognizing keratin 10, which binds to less than 2% of the cultured keratinocytes was used as a negative control (figure 10A). Relative DNA content obtained from bivariate analysis gave similar results to single parameter measurements, despite the additional use of antibody and the increased number of incubation and wash-steps. Furthermore, the technique is reproducible and statistically reliable results were obtained.

In conclusion, we have showed that the Ki-67 antigen is present in normal and hyperproliferative epidermis *in vivo*, and in cultured human keratinocytes. Quantification of the results obtained with the immunoperoxidase technique was often hampered by the fact that cytoplasmic staining interferes with the nuclear staining, in particular when cryostat sections derived from normal skin were used. Furthermore, we were able to measure Ki-67 binding in cultured keratinocytes flow cytometrically, but not in cell suspensions derived from skin biopsies. Optimization of the flow cytometric technique was carried out with activated human peripheral lymphocytes, showing that ethanol fixation is preferred over other fixatives.

4. References

1. Gerdes J, Schwab U, Lemke H, Stein H: Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31:13-20, 1983

2. Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H: Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 133:1710-1715, 1984

3. Tan EM: Autoantibodies to nuclear antigens (ANA): their immunobiology and medicine. Adv Immunol 33:167-240, 1982

4. Miyachi K, Fritzler MJ, Tan EM: Autoantibody to a nuclear antigen in proliferating cells. J Immunol 121:2228-2234, 1978

5. Klein G, Steiner M, Wiener F, Klein E: Human leukemia-associated anti-nuclear reactivity. Proc Natl Acad Sci USA 71:685-689, 1974

6. Celis JE, Madsen P, Celis A, Nielsen HV, Gesser B: Cyclin (PCNA, auxiliary protein of DNA polymerase δ) is a central component of the pathway(s) leading to DNA replication and cell division. FEBS Lett 220:1-7, 1987

7. Ogata K, Kurki P, Celis JE, Nakamura RM, Tan EM: Monoclonal antibodies to a nuclear protein (PCNA/cyclin) associated with DNA replication. Exp Cell Res 168:475-486, 1987

8. Papadopoulos T, Pfeifer GP, Hoppe F, Drahovsky D, Müller-Hermelink H-K: Proliferation-associated expression of DNA methyltransferase in human embryonic lung cells. Virchows Archiv B Cell Pathol 56:371-375, 1989

9. Neubauer A, Serke S, Siegert W, Kroll W, Musch R, Huhn D: A flow cytometric assay for the determination of cell proliferation with a monoclonal antibody directed against DNA-methyltransferase. Brit J Haematol 72:492-496, 1989

10. Boezeman JBM, Bauer FW, de Grood RM: Flow cytometric analysis of the recruitment of G_0 cells in human epidermis in vivo following tape stripping. Cell Tissue Kinet 20:99-107, 1987

11. Bauer FW, Crombag NHCMN, de Grood RM, de Jongh GJ: Flow cytometry as a tool for the study of cell kinetics in epidermis. Investigations on normal epidermis. Brit J Dermatol 102:629-639, 1980

12. Bauer FW, Boezeman JBM: Flow cytometric methods in human skin with respect to cell cycle kinetics. in: Psoriasis: cell proliferation. Edited by Wright NA, Camplejohn RS. Edinburgh, Churchill Livingstone, pp 104-116, 1983

13. Rheinwald JG, Green H: Serial cultivation of strains of human keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331-344, 1975

14. Kurki P, Ogata K, Tan EM: Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. J Immunol Methods 109:49-59, 1988

15. Palutke M, KuKuruga D, Tabaczka P: A flow cytometric method for measuring lymphocyte proliferation directly from tissue culture plates using Ki-67 and propidium iodide. J Immunol Methods 105:97-105, 1987

16. Brons PPT, Pennings AHM, Haanen C, Wessels HMC, Boezeman JBM: Simultaneous measurement of DNA content and cell-surface immunofluorescence of human bone marrow cells using a single laser flow cytometer. Cytometry 11:837-844, 1990

17. Sasaki K, Murakami T, Kawasaki M, Takahashi M: The cell cycle associated change of the Ki-67 reactive nuclear antigen expression. J Cell Physiol 133:579-584, 1987

18. Gelfant S: The cell cycle in psoriasis: a reappraisal. Brit J Dermatol 95:577-590, 1976

19. Weinstein GD, McCullough JL, Ross PA: Cell kinetic basis for pathophysiology of psoriasis. J Invest Dermatol 85:579-583, 1985

20. Weinstein GD, McCullough JL: Cytokenetics in diseases of epidermal hyperplasia. A Rev Med 24:345-352, 1973

21. Allegra F, De Panfilis G: An in vivo method of studying the kinetics of cell proliferation in normal human epidermis. Acta Derm Venereol (Stockh) 54:87-90, 1974

22. Rijzewijk JJ, Bauer FW, Boezeman JBM, Happle R, Mier PD: Recruitment of quiescent (G_0) cells following epidermal injury is initiated by activation of the phosphoinositol cycle. J Invest Dermatol 90:44-47, 1988

23. Larsen JK, Frentz G, Møller U, Christensen IJ: A method for flow cytometric cell cycle analysis of normal and psoriatic human epidermis based on a detergent/citric acid technique for suspension of nuclei. Virchows Arch B Cell Pathol 48:247-259, 1985

24. Stough DR, Burns ER, Mallory SB, Pipkin JL, Hinson WG: Modification of a trypsin-detergent method for DNA flow cytometry of human epidermis. Cytometry 10:90-93, 1989

CHAPTER V. CHANGES IN INTRACELLULAR pH AS AN EARLY FUNCTIONAL MARKER OF KERATINOCYTE ACTIVATION

This chapter was based on the following publications:

Piet van Erp, Monique Jansen, Gijs de Jongh, Jan Boezeman, Joost Schalkwijk, Flow cytometric measurement of intracellular pH in cultured human keratinocytes using carboxy-SNARF-1. in: Advances in analytical cellular pathology, Eds. Burger G, Oberholzer M, Vooijs GP. Amsterdam, Excerpta Medica, 1990, pp 131-132

Piet EJ van Erp, Monique JJM Jansen, Gijs J de Jongh, Jan BM Boezeman, Joost Schalkwijk, Ratiometric measurement of intracellular pH in cultured human keratinocytes using carboxy-SNARF-1 and flow cytometry. Cytometry 12:127-132, 1991

CHAPTER V. CHANGES IN INTRACELLULAR pH AS AN EARLY FUNCTIONAL MARKER OF KERATINOCYTE ACTIVATION

1. Introduction 83
2. Methods & Materials 84
2.1. Cell Culture (84)
2.2. Isolation of Polymorphonuclear Leukocytes (84)
2.3. Solutions and Sample Preparation (84)
2.4. Spectrofluorimetry (85)
2.5. Staining for Relative DNA Measurements (85)
2.6. Flow Cytometry (85)
3. Results and Discussion 87
4. References 93

1. Introduction

Cellular metabolism and function depends critically on maintenance of the intracellular (cytoplasmic) pH (pH_i) within narrow limits. Measurement of pH_i can therefore be used to obtain information about important processes in the cell, such as cell division[1,2]. In contrast to other methods to detect changes in pH_i, such as weak acid distribution and microelectrodes, the use of pH-sensitive fluorochromes in combination with flow cytometry (FCM) allows analysis of heterogeneous cell populations on a single cell basis.

In normal human epidermis not all of the germinative cells are cycling. We hypothesize that cell production is regulated by a $G_0 \rightarrow G_1$ induction of germinative cells[3]. Methods to detect cycling cells, using the MAb Ki-67[4,5], anti-cyclin[6], or anti-BrdUrd[7,8] all have their own limitations (see also chapter III and IV). Our aim was the development of a flow cytometric method to measure pH_i in quiescent and cycling keratinocytes using cultured keratinocytes as a model. We have tested the recently developed pH-sensitive dye carboxy-seminaphthorhodafluor-1 (SNARF-1)[9] as a possible candidate to measure growth fractions in epidermis, and compared it with 2'7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) and 2,3-dicyanohydroquinone (DCH) with respect to emission spectra, resolution, pH range, and stability of cellular fluorescence.

2. Methods & Materials

2.1. Cell Culture

Human neonatal foreskin keratinocytes were cultured on 3T3 feeder cells according to Rheinwald and Green[10] as described in chapter II at p. 33. EDTA treated, trypsinized keratinocytes from the first 1-2 passages were used in the experiments. Cells (more than 95% keratinocytes, using an anti-keratin antibody as a marker) were either kept on ice for immediate use or fixed in 70% ice-cold ethanol and stored at -20°C. In some experiments 0.2% v/v fetal bovine serum was used instead of 6% v/v fetal bovine serum.

2.2. Isolation of Polymorphonuclear Leukocytes

Polymorphonuclear leukocytes (PMNs) were isolated from 1ml heparinized whole human blood according to a standard procedure[11]. Yield was 1.7-2.3x10^6 PMNs. The cells were kept on ice until use.

2.3. Solutions and Sample Preparation

Stock solutions of 10 mg/ml 1,4-diacetoxy-2,3-dicyanobenzene (ADB, Calbiochem, La Jolla, CA, USA), 1 mM BCECF/AM (Molecular Probes, Inc., Eugene, OR, USA) and 1 mM SNARF-1/AM (Molecular Probes, Inc., Eugene, OR, USA) were prepared in dimethylsulphoxide, and stored at 4°C. ADB is the esterified derivative of DCH, which is enzymatically hydrolysed inside cells. BCECF and SNARF-1 are introduced into cells in the form of acetoxymethyl (AM) esters, and undergo enzymatic hydrolysis in cells to yield the free fluorescent dye. Aliquots of 10^6 keratinocytes were washed with PBS (Biochrom KG, Berlin, FRG) and resuspended in 1ml PBS. The cells were loaded with 10 μl stock dye solution for 15 min at 37°C. Spectrofluorimetric and flow cytometric measurements in general were carried out in 40 mM Hepes buffer containing 115 mM NaCl and 1 mM $MgCl_2$ or in 40 mM Hepes buffer containing 115 mM KCl and 1 mM $MgCl_2$. A stock solution of 1 mM nigericin (Molecular Probes, Inc., Eugene, OR, USA) was prepared in ethanol, and stored at 5°C. The nigericin calibration experiments were carried out in K^+/Hepes buffer of known pH. Prior to flow cytometric measurement, 10 μl nigericin stock solution was added to 1ml cell suspension.

2.4. Spectrofluorimetry

Samples for spectrofluorimetry, containing $1.0x10^6$ freshly prepared cells (keratinocytes or PMNs), were loaded with ADB, BCECF/AM or SNARF-1/AM as described above. The cells were washed with PBS and finally resuspended in phosphate-buffers of known pH (range 6-8). After lysing the cells by sonication for a few seconds, and centrifugation, the emission spectra of DCH, BCECF, and SNARF-1 in the supernatant were recorded using a Perkin-Elmer LS5 fluorescence spectrometer. Excitation at 351 nm for DCH and 488 nm for both BCECF and SNARF-1 was chosen to match the excitation wavelengths available from the argon ion laser of the flow cytometer.

In experiments to test dye leakage from cells, aliquots of loaded cells were washed with ice-cold PBS. At different time intervals a sample was centrifuged and the pellet was washed once. The fluorescence intensity of the pooled supernatants was measured.

To test whether most of the dye is "free" in the cytoplasm, samples of loaded cells were washed three times with ice-cold PBS, lysed either by sonication or addition of 0.1% v/v triton X-100, and centrifuged. The pellet was washed once and the fluorescence intensity of the pooled supernatants were measured as an estimate of "free" dye. The fluorescence intensity of the resuspended pellet was a measure for "bound" dye.

2.5. Staining for Relative DNA Measurements

Between $0.5\text{-}1.0x10^6$ ethanol-fixed cells were used for further preparation. The cells were resuspended in 400 μl PBS containing 20 μg/ml PI (Calbiochem, San Diego, CA, USA) and incubated for 15 min with 50 μl 1% w/v RNAse (Sigma, St. Louis, MO, USA). Prior to flow cytometric analysis cell suspensions were filtered through gauze (mesh 50 μm, V. Wijk, Santpoort, The Netherlands).

2.6. Flow Cytometry

Excitation conditions and optical emission filters used for pH_i measurements are given in table I. The area of the fluorescence signals and the right angle scatter (RAS) were recorded in list mode using a flow cytometer 50H (Ortho Instruments, Westwood, CA, USA) equipped with a 5-Watt Argon ion laser.

Tabel I Excitation conditions and optical emission filters used for pH_i measurements

SNARF-1	DCH	BCECF
488nm, 400mW	UV, 80mW	488nm, 400mW
Dichroic 600nm	Dichroic 460nm	Dichroic 580nm
Band 564-606nm	Band 390-440nm	Band 515-530nm
Band 608-643nm	Band 515-545nm	Band 608-643nm

The 488 nm line of the 5-Watt Argon ion laser was used for excitation, when cells were stained with propidium iodide for relative DNA content. A highpass filter RG630 nm for red fluorescence was used. If possible, 5×10^4 cells were measured at a flow rate of approximately 300 cells per second. Both area and peak value of the red fluorescence signal were recorded in list mode. The ratio area:peak discriminates between artefacts due to doublets of diploid cells and real single tetraploid (or late S) cells, when intact cells are used[12].

Table II Comparison of SNARF-1 with BCECF and DCH with respect to dye leakage, background fluorescence, fluorescence stability, and binding to cellular structures[a]

Parameter	SNARF-1	DCH	BCECF
Binding to cellular structures	20%	10%	5%
Range	1 5 pH unit	>1 5 pH unit (pH 6-8)	>1 5 pH unit
Resolution	0 3 pH unit (pH 7-8)		0 4 pH unit
Background fluorescence	low	high	low
Leakage	2 h <10%	0 5 h 50%	1 h <10%

[a]Similar results were obtained for keratinocytes and PMNs

3. Results and Discussion

We have tested the recently-developed dye SNARF-1 and compared it with other commonly used fluorescent pH-indicators such as BCECF[2,13] and DCH[1,14], with respect to stability of cellular fluorescence, cytoplasmic specificity, and dye leakage. A flow cytometric method, using SNARF-1, has been applied to obtain the first measurements of pH_i in human keratinocytes. We show here that the use of a dye which changes colour with pH (rather than merely altering intensity) overcomes the technical problem that the population is heterogeneous with respect to size and metabolic activity (e.g. non-specific esterase activity). The ratio of emission intensity at two wavelengths can then be used as an indication of the colour change[15], and thus as a measure for pH_i. In many experiments peripheral blood PMNs were investigated in parallel in order to evaluate the effect of trypsinisation of cultured keratinocytes, and because PMNs have been used before for pH-measurements[16,17,18].

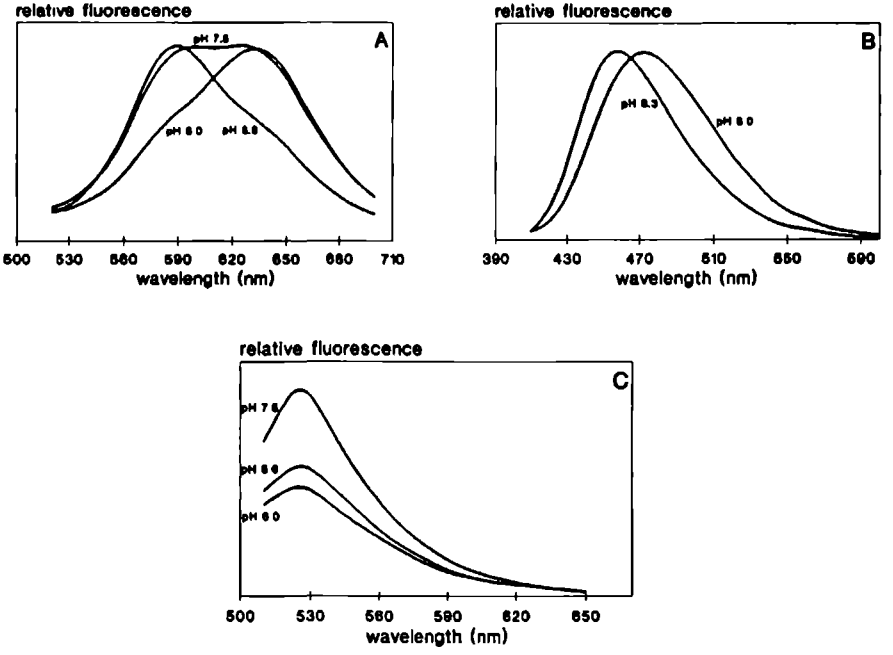


Figure 1 Emission spectra of SNARF-1 (A), DCH (B), and BCECF (C). Dye-loaded cells were lysed by sonication, and the emission spectra recorded using a fluorescence spectrometer.

Characteristics of SNARF-1, and a comparison with DCH and BCECF are summarized in table II. In our hands, DCH proved difficult to use, primarily because UV excitation is not routinely used at our department, but also because DCH is rapidly released (leakage 50% within 30 min) from keratinocytes or PMNs, and it gives a relatively high background fluorescence. In contrast, leakage of SNARF-1 and BCECF from cells was negligible over the period between dye-loading and flow cytometric measurement (in general less than 1 h). Furthermore, visible-wavelength excitation is much easier to use, and gives very stable fluorescence for both SNARF-1 and BCECF. A disadvantage for SNARF-1 is that about 20% of the cell-associated dye is bound or trapped in cell organelles. Still, the major part of SNARF-1 is found in the cytoplasm. Overall, the fluorochromes SNARF-1 and BCECF were found to have similar physical characteristics, and were preferred over DCH in this respect. No differences were found between keratinocytes and PMNs.

Spectral characteristics of SNARF-1 are shown in figure 1A. In contrast to BCECF, and many other fluorescent pH indicators, SNARF-1 was reported to have a pH-dependent shift in emission wavelength[9]. Using an excitation wavelength of 488 nm, which resembles the main visible line of the argon laser, the spectrum peaks at about 580 nm when dye-loaded cells were lysed in a buffer of pH 6. At pH 8 the top is at about 630 nm. DCH (figure 1B) shows a comparable emission shift. However, using DCH the excitation wavelength had to be set at 351 nm. With increasing pH the emission spectrum shifts from 460 nm at pH 6 to 480 nm at pH 8. In contrast, the emission spectrum of BCECF (figure 1C) does not shift with pH. The spectral characteristics of fluorescein

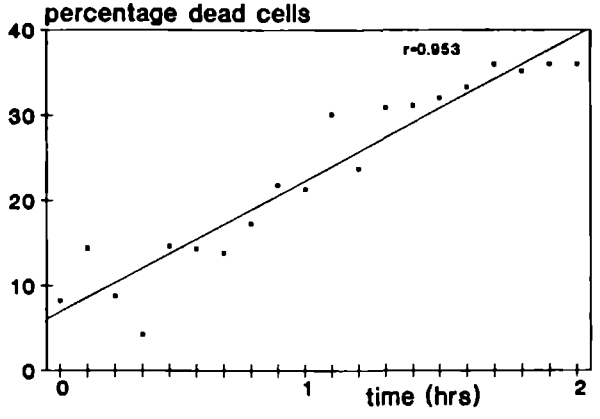


Figure 2 Percentage dead cells (low fluorescence, low RAS) as a function of time at room temperature after dye-loading (BCECF).

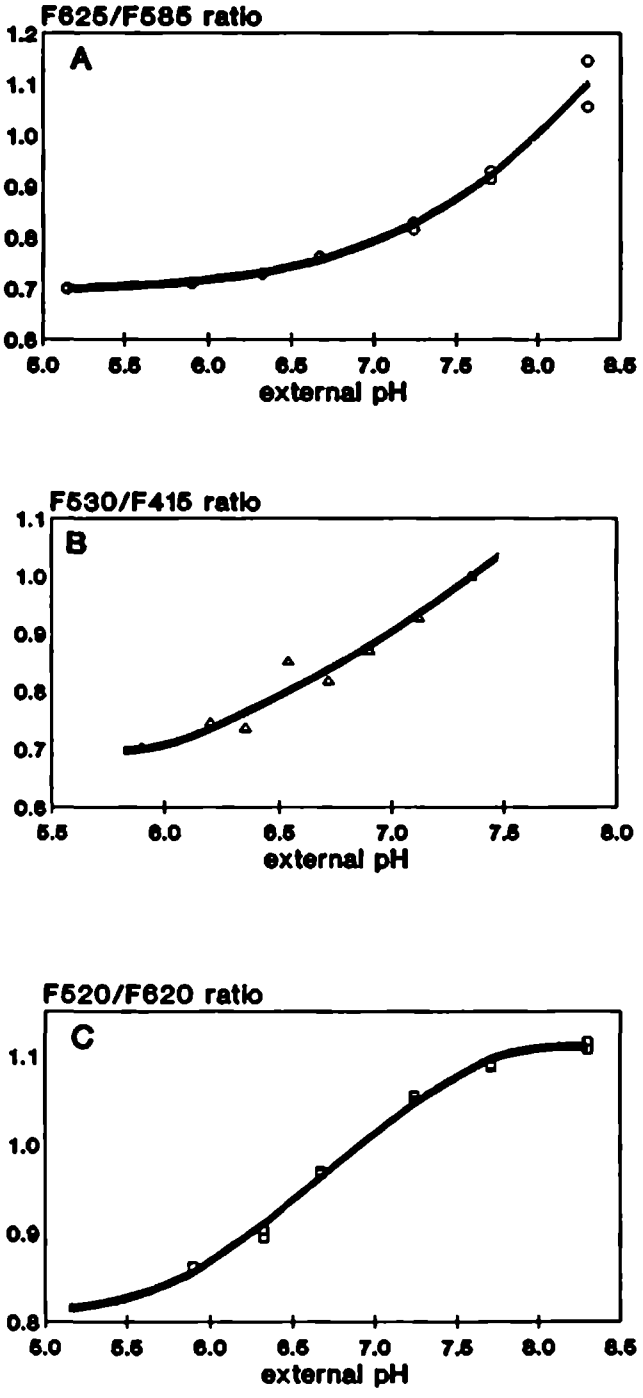


Figure 3 Calibration curves for keratinocytes incubated with SNARF-1/AM (A), ADB (B) and BCECF/AM (C) and measured flow cytometrically.

derivatives, such as BCECF, are well known. Both the excitation and emission spectra of BCECF are pH-sensitive with relative pH-sensitivity maximal at the peak fluorescence wavelengths (488 nm for excitation and 525 nm for emission), and minimal in the tails of the curves[19].

Musgrove et al[13] have used this knowledge to measure the pH dependence of BCECF using an excitation wavelength of 488 nm, and emission wavelengths of 520 nm and 620 nm. The fluorescence intensity at 620 nm depends mainly on cell size and dye load, and offers a means of correction.

For all three fluorochromes, the ratio of fluorescence intensities at two wavelengths was recorded and used as a measure of pH_i by reference of pH in the presence of the proton ionophore nigericin. Nigericin can be cytotoxic for cells. In order to minimize the percentage of dead cells, flow cytometric measurements were performed within 30 minutes after the addition of nigericin. Incubations with nigericin were between 10 and 30 minutes. Dead cells could be recognized flow cytometrically by very low fluorescence and relatively low-scatter, and were omitted from the analysis The percentage of dead cells

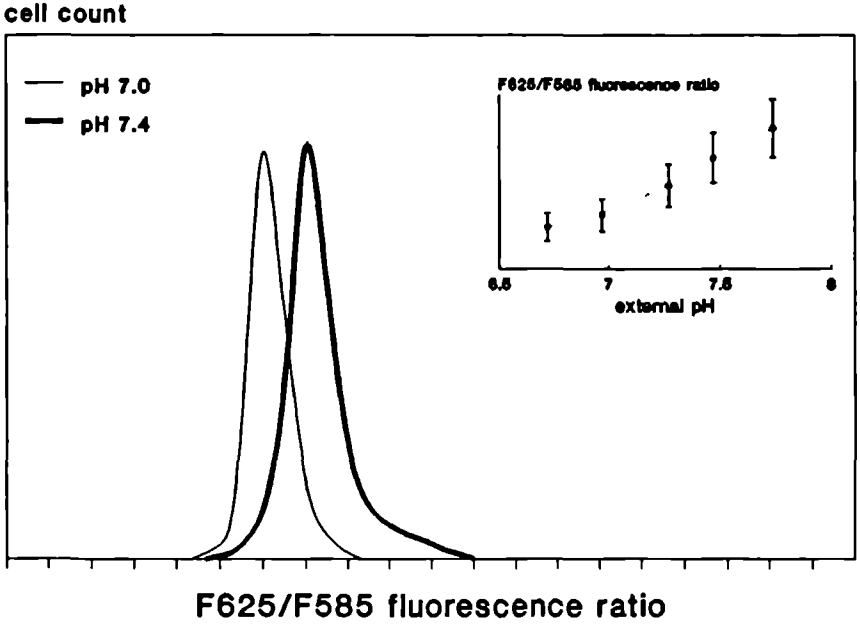


Figure 4 Fluorescence ratio histograms of keratinocytes loaded with SNARF-1 in the presence of nigericin The insert shows a total calibration curve

also increased with time after dye-loading, but did not exceed 35% up to 2 h at room temperature (figure 2). This increase in dead cells with time was independent of whether SNARF-1 or BCECF were used, suggesting that the incubation conditions rather than the dye itself caused the cell death. Typical pH-calibration curves for all three fluorochromes are shown in figure 3. From these apparent pH_i values could be calculated from the measured fluorescence ratio of a given sample without nigericin (data not shown). SNARF-1 has a linear traject between pH 7 and pH 8. In the case of DCH and BCECF the curves were linear between pH 6.5 and pH 7.5. Comparison of maximum resolution, estimated from fluorescence ratio histograms in the presence of nigericin[13] revealed better resolution for SNARF-1 in the range pH 7-8 (figure 4). However, at lower pH BCECF gave comparable or even better resolution.

To illustrate that pH-measurements were independent of dye load, in figure 5 dye-concentration was plotted against fluorescence ratio and

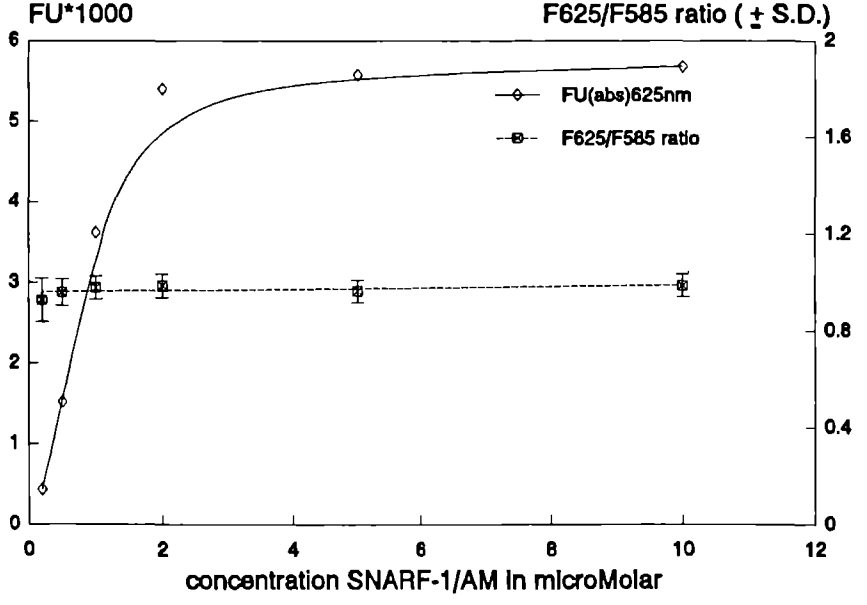


Figure 5 SNARF-1/AM concentration plotted against fluorescence ratio and fluorescence intensity (625 nm), illustrating that the fluorescence ratio is independent of dye content.

fluorescence intensity (625 nm). The average fluorescence intensity per cell was increasing with dye content. However, when fluorescence ratio was plotted against dye-concentration the result was a horizontal line.

The fact that SNARF-1 offers the possibility of ratiometric measurements on the basis of a real emission shift, and had superior resolution for the pH range we are interested in (pH 7.0-7.5), made it the fluorochrome of choice. Using SNARF-1 we found that keratinocytes cultured under low serum conditions (0.2%) contain a higher proportion of cells with relatively low pH_i

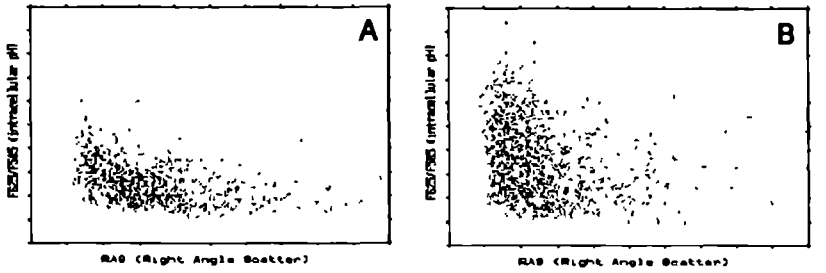


Figure 6 Typical bivariate plots of scatter (RAS) on the x-axis and fluorescence ratio on the y-axis for keratinocytes cultured in medium containing 0.2% calf serum (A) and 6% calf serum (B).

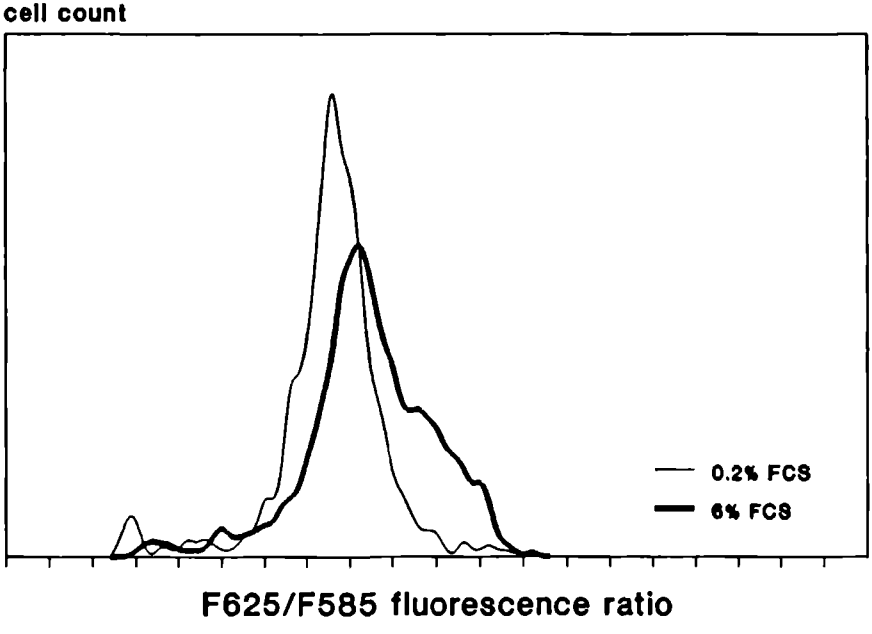


Figure 7 Fluorescence ratio histograms of SNARF-1-loaded keratinocytes derived from cultures supplemented with 0.2% and 6% calf serum

compared to high serum cultures (6%) (figure 6A and 6B, respectively). These results were obtained 24 hrs after switching to medium containing 0.2% fetal calf serum. In contrast to many other cell types, keratinocytes with relative high pH seem to have a low RAS signal. This can be explained by the fact that a subpopulation of keratinocytes *in vitro*, and also *in vivo*, has irreversibly committed itself to terminal differentiation; they are destined to die, and *in vivo* will form the dead surface layer of the skin (stratum corneum). The differentiating cells undergo dramatical morphological changes, including increased size and appearance of various intracellular lamellar bodies. These changes will increase the RAS signal. However, these cells are with certainty not proliferative, and should therefore have a relatively lower pH.

The pH changes were followed by changes in relative DNA content. At the first day in medium containing 0.2% foetal calf serum, 52.4% (S.D. ±0.5, n=4) of the cells were in S, G2, and M-phase of the cell cycle (comparable to values obtained for keratinocytes cultured in medium containing 6% fetal calf serum). At day three this percentage was decreased to 36.8% (S.D. ±1.3, n=5). In figure 7 fluorescence ratio histograms of the data presented in figure 6 are shown. When keratinocytes are cultured in 6% foetal calf serum, two subpopulations of keratinocytes on the basis of pH_i can be detected. Although further experiments are required to test the hypothesis that the subpopulation of cells with relatively high pH_i coincides with the cycling cell population, the findings presented above suggest that flow cytometric measurement of pH_i can be an early functional proliferation marker for human keratinocytes.

4. References

1 Gillies RJ, Cook J, Fox MH, Giuliano KA. Flow cytometric analysis of intracellular pH in 3T3 cells Am J Physiol 253 C121-C125, 1987

2. Musgrove E, Seaman M, Hedley D Relationship between cytoplasmic pH and proliferation during exponential growth and cellular quiescence Exp Cell Res 127.65-75, 1987

3 Boezeman JBM, Bauer FW, de Grood RM· Flow cytometric analysis of the recruitment of G_0 cells in human epidermis *in vivo* following tape stripping Cell Tissue Kinet 20 99-107, 1987

4. Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H· Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 133.1710-1715, 1984

5 Van Erp PEJ, De Mare S, Rijzewijk JJ, van de Kerkhof PCM, Bauer FW A sequential double immunoenzymic staining procedure to obtain cell kinetic information in normal and hyperproliferative epidermis Histochem J 21 343-347, 1989

6 Kurki P, Ogata K, Tan EM Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry J Immunol Methods 109 49-59, 1988

7 Pallavicini MG, Summers LJ, Dolbeare FD, Gray JW Cytokinetic properties of asynchronous and cytosine arabinoside perturbed murine tumors measured by simultaneous bromodeoxyuridine/DNA analysis Cytometry 6 602-610, 1985

8 Van Erp PEJ, Brons PPT, Boezeman JBM, de Jongh GJ, Bauer FW A rapid flow cytometric method for bivariate bromodeoxyuridine/DNA analysis using simultaneous proteolytic enzyme digestion and acid denaturation Cytometry 9 627-630, 1988

9 Whitaker JE, Haugland RP, Prendergast FG Seminaphto-fluoresceins and -rhodafluors dual fluorescence pH indicators Biophys J 53 197a, 1988

10 Rheinwald JG, Green H Serial cultivation of strains of human keratinocytes the formation of keratinizing colonies from single cells Cell 6 331-344, 1975

11 Trush MA, Wilson ME, Van Dyke K The generation of chemiluminescence (CL) by phagocytic cells In Methods in Enzymology, Vol LVII Ed DeLuca MA Academic Press, New York, 1978, pp 115 198

12 Bauer FW, Boezeman JBM Flow cytometric methods in human skin with respect to cell cycle kinetics In Psoriasis Cell Proliferation, Eds Wright NA, Camplejohn RS Churchill Livingstone, New York, 1983, pp 104 115

13 Musgrove E, Rugg C, Hedley D Flow cytometric measurement of cytoplasmic pH a critical evaluation of available fluorochromes Cytometry 7 347-355, 1986

14 Valet G, Raffael A, Moroder L, Wunsch E, Ruhenstroth Bauer G Fast intracellular pH determination in single cells by flow cytometry Naturwissenschaften 68 265 266, 1981

15 Shapiro HM Parameters and probes In Practical Flow Cytometry Alan R Liss, Inc, New York, 1988, pp 115 198

16 Grinstein S, Furuya W Cytoplasmic pH regulation in activated human neutrophils Effects of adenosine and pertussis toxin on Na^+/H^+ exchange and metabolic acidification Biochim Biophys Acta 889 301 309, 1986

17 Rothe G, Valet G Phagocytosis, intracellular pH, and cell volume in the multifunctional analysis of granulocytes by flow cytometry Cytometry 9 316-324, 1988

18 Weisman SJ, Punzo A, Ford C, Sha'afi RI Intracellular pH changes during neutrophil activation Na^+/H^+ antiport J Leukocyte Biol 41 25 32, 1987

19 Graber ML, DiIillo DC, Bradford LF, Pastoriza Munoz E Characteristics of fluoroprobes for measuring intracellular pH Anal Biochem 156 202-212, 1986

CHAPTER VI. APPLICATION OF CYTOMETRIC METHODS TO STUDY GROWTH AND DIFFERENTIATION IN NORMAL AND PSORIATIC EPIDERMIS

This chapter was based on the following publications:

Piet EJ van Erp, Hennie Groenendal, Franz W Bauer, Joris J Rijzewijk, Growth fraction in epidermal skin disorders determined by the monoclonal antibody Ki-67. J Invest Dermatol 89:313, 1987

Piet EJ van Erp, Saskia de Mare, Joris J Rijzewijk, Peter CM van de Kerkhof, Franz W Bauer, A sequential double immunoenzymic staining procedure to obtain cell kinetic information in normal and hyperproliferative epidermis. Histochem J 21:343-347, 1989

Piet EJ van Erp, Joris J Rijzewijk, Jan BM Boezeman, John Leenders, Saskia de Mare, Peter CM van de Kerkhof, Frans CS Ramaekers, Franz W Bauer, Flow cytometric analysis of epidermal subpopulations from normal and psoriatic skin using monoclonal antibodies against intermediate filaments. Am J Pathol 135:865-870, 1989

Piet EJ van Erp, Jan BM Boezeman, Paul PT Brons, Cell cycle kinetics in human skin by in vivo administration of iododeoxyuridine and application of a differentiation marker. Manuscript in preparation.

CHAPTER VI. APPLICATION OF CYTOMETRIC METHODS TO STUDY GROWTH AND DIFFERENTIATION IN NORMAL AND PSORIATIC EPIDERMIS

1. Introduction 97
2. Methods & Materials 99
2.1. Patient Material and Skin Sampling (99)
2.2. Immunoperoxidase Staining (99)
2.3. Double Immunoenzymatic Staining Method (100)
2.4. Preparation and Staining of Cell Suspensions (101)
2.5. In Vivo Labelling of Normal Skin with IdUrd (101)
3. Results 102
3.1. Estimation of the Growth Fraction (102)
3.2. Double Immunoenzymatic Staining Method (105)
3.3. Flow Cytometric Analysis Using Monoclonal Antibodies Against Intermediate Filaments (108)
3.4. Incorporation of IdUrd *in vivo*. (109)
4. Discussion 110
5. References 115

1. Introduction

Psoriasis is one of the most common of all skin diseases, and has been subject to many investigations over the last century. It is well established that psoriasis has a genetic basis. The cause of the disease and the mechanisms involved at the cellular and molecular level are not known. A better understanding of this puzzling disease would also improve our basic knowledge of growth regulation in normal epidermis. Many investigators have claimed to have found the major defect in psoriatic epidermis or have reported possible defective pathways. Alterations in many or all of the parameters of cell division[1,2,3] were amongst the first abnormalities reported. Decreased cyclic AMP levels in lesional psoriatic skin[4] were the basis of a concept first proposed by Voorhees and Duell[5] which involved second messenger systems. In the early nineteen eighties fucosylated glycoproteins, possibly acting as

plasma membrane receptors, were found to be abnormal in psoriatic lesions and also in uninvolved skin[6,7,8,9]. In 1983 it was proposed by Forster et al that phospholipase A_2 activity was raised in the uninvolved epidermis of psoriasis[10]. These authors claimed that their observation was the first demonstration of an abnormality in the uninvolved untreated psoriatic epidermis which could account for the pathology of the disease. These findings also link two of the major characteristics of the clinical lesion, epidermal hyperproliferation and inflammatory changes. A further fact to be considered when discussing elevated levels of epidermal phospholipase A_2 activity are reports by Van de Kerkhof and Van Erp[11], and by Tucker et al[12] that calmodulin levels are elevated in psoriatic skin. Tucker et al even found that active calmodulin levels were increased in the lesion-free epidermis of psoriasis. There is thus the possibility that increased phospholipase A_2 activity is the result of an abnormality in the regulation of calmodulin activity and in calcium-dependent second messenger systems. Recently it has been shown that transforming growth factor-alpha (TGF_α) is overexpressed in psoriatic epidermis as determined by immunohistochemistry with TGF_α-specific monoclonal antibodies (MAb) on tissue sections[13] or by the abundance of TGF_α-specific mRNA and protein in epidermal extracts[14]. Epidermal growth factor (EGF)-receptors are also overexpressed in psoriatic epidermis as described by Nanney et al[15]. This overexpression of the EGF-receptor and its ligand TGF_α suggest an obvious mechanism for increased keratinocyte proliferation involving signal transduction by protein tyrosine phosphorylation. In psoriatic epidermis, altered protein tyrosine kinase activity has been detected[16]. From these studies it is suggested that a primary epidermal abnormality, if it exists, could be in one of the signal transduction pathways of epidermal cells. These findings imply that we can be rather sure that psoriasis is, in molecular terms, a disorder of communication; the individual cell seems remarkably healthy, but the normal pattern of growth is disturbed. Obviously, search for factors involved in the autocrine and paracrine communication systems, investigations towards the role of extracellular matrix, and studies of cell-cell communication, should have high priority. However, to be able to clarify the different communication routes involved in epidermal growth and differentiation, we also have to improve our knowledge with respect to the heterogeneity of the

epidermis, and which cells or cell compartments are actually involved.

Therefore, investigations presented in this chapter basically can be divided in two groups. Firstly, we have applied the techniques described in the previous chapters to normal and psoriatic epidermis. Questions were:

Can we divide the epidermis in different compartments on the basis of cell-type-specific markers?

What are the pool-sizes of the different epidermal compartments?

Can we define more specifically the cell cycle abnormalities in psoriasis?

Secondly, we have used the results obtained from psoriatic and normal epidermis to gain more information on the process of growth control in normal epidermis.

2. Methods & Materials

2.1. Patient Material and Skin Sampling

We selected patients with chronic plaque psoriasis, atopic dermatitis, and some other less common diseases, such as Darier's disease, benign familial chronic pemphigus (BFCP), ichthyosis vulgaris, and X-linked recessive ichthyosis. All skin diseases were characterized by some degree of epidermal hyperproliferation and/or dyskeratinization. Some of the psoriatic patients were treated with either corticosteroids or methotrexate, whereas the others had received no therapy for at least one month. All patients with atopic dermatitis were treated with topical corticosteroid. In the untreated psoriatic patients both lesional and distant clinically uninvolved skin were biopsied. The healthy control subjects were paid volunteers with no sign or history of skin disease. In the case of *in vivo* IdUrd labelling normal skin was acquired from consenting patients with malignant lymphoma.

Microbiopsy specimens (3 mm in diameter and about 0.2 mm thick) were taken freehand using a razor blade in conjunction with a metal guard as described in chapter II at p. 33. Normal skin 3-6 h after *in vivo* IdUrd pulse labelling was derived from surgical specimens.

2.2. Immunoperoxidase Staining

The standard technique for staining with MAb Pab601 on cryostat sections is given in chapter II and Ki-67 staining was performed as described in chapter IV.

2.3. Double Immunoenzymatic Staining Method

Skin biopsies were washed in PBS and floated, dermal face downward, in a 5 ml glass bottle containing 2 ml Medium 199 with Hanks' salts, 25 mM Hepes, L-glutamine (Gibco, Paisley, Scotland) and 135 μg/ml BrdUrd (Serva, Heidelberg, FRG) for 1 h at 37°C in air at 1 atmosphere effective pressure. The incubation was stopped by transferring the biopsies to a petri dish containing ice-cold PBS.

Biopsies were embedded in Tissue-Tek II O.C.T. compound (Miles Scientific, Naperville, USA) in a cryostat at -20°C. Sections of 8 μm were cut and fixed directly in acetone-ether (60/40 v/v) for 10-20 min. Fixed and air-dried sections were either kept at -20°C or used directly.

The double labelling procedure as it was routinely used is given below. Dilutions for each antibody were optimised, and control experiments were carried out as described in the result section. After 3-5 min incubation in PBS containing 0.01% Tween 80, the sections were incubated with Ki-67 (Dakopatts, Copenhagen, Denmark) diluted 1:20 in PBS for 30 min at room temperature in a moist chamber, washed with PBS and subsequently incubated for 30 min with RAMPO (Dakopatts, Copenhagen, Denmark) at a dilution of 1:25 in PBS containing 5% human AB serum. After washing with PBS the bound peroxidase was developed with the AEC/H_2O_2 reaction for 20 min. The sections were rinsed in distilled water and hydrolysed for 10 min in 4 N HCl at room temperature in order to partially denature DNA. Binding of the monoclonal anti-BrdUrd requires regions of single-stranded DNA. Exact denaturation conditions were established previously (Chapter III). After two washes with PBS the sections were incubated for 30 min with anti-BrdUrd (Becton & Dickinson, Mountain View, USA), diluted 1:10 in PBS containing 0.5% Tween 20. The sections were washed with TRIS-buffer, (pH 9.1) and incubated for 30 min with an alkaline phosphatase-conjugated rabbit anti-mouse antibody (Dakopatts, Copenhagen, Denmark) at dilution of 1:10 in TRIS buffer containing 1% human AB serum. After washing with TRIS buffer, the bound alkaline phosphatase was visualized with naphthol AS-MX phosphate and Fast Blue RR as a chromogen for 10 min. Sections were finally rinsed in distilled water, mounted in glycerine gelatine and positive cells counted per mm length of section.

2.4. Preparation and Staining of Cell Suspensions

Cell suspensions were prepared by trypsinization of biopsies according to Bauer and Boezeman[17], and stained with MAb against intermediate filaments. Experimental conditions and dilutions of antibodies were as given in chapter II.

2.5. In Vivo Labelling of Normal Skin with IdUrd

Pulse labelling was performed by a 15 min infusion with 200 mg IdUrd. A biopsy was taken 3-6 h after labelling. Single cell suspensions were prepared by trypsinization as in section 2.4., and fixed in 70% ethanol. IdUrd was visualized with anti-IdUrd MAb HN-IU after a one step combined pepsin/HCl denaturation protocol (Chapter III).

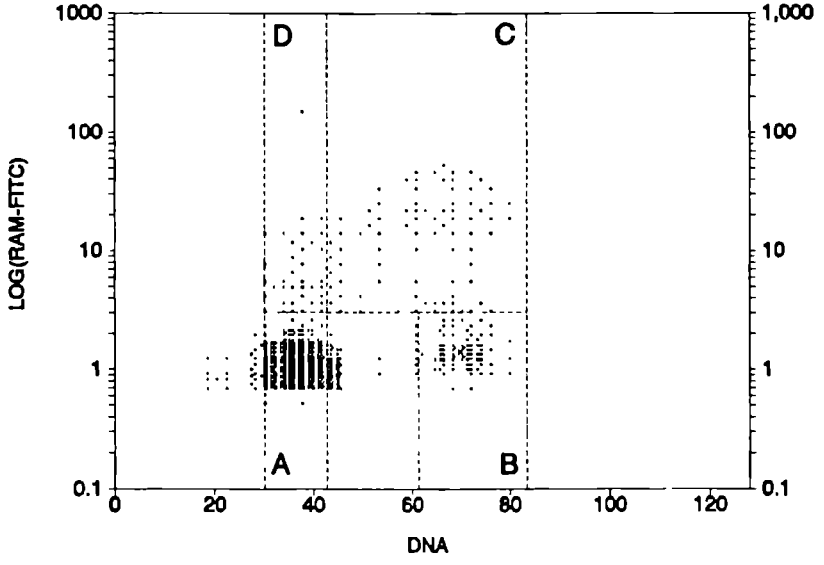


Figure 1 Example of IdUrd/DNA measurement. Plots are divided into 4 windows. A: G0G1 cells. B: G2M cells. C: IdUrd labelled cells. D: IdUrd labelled cells excluded from calculation.

Two parameter FCM of DNA versus BrdUrd/IdUrd, allows assessment of the number of cells in S-phase (N_S) and the duration of the S-phase (T_S) even from one single sample[18]. The calculation of T_S is then based on the assumption that there is a linear increase in mean relative DNA content of the IdUrd-labelled S-phase cells in time. At time 0 after IdUrd pulse labelling, the mean DNA content of the IdUrd-labelled S-phase cells is in the middle of the interval

between the unlabelled diploid G_0/G_1 cell population and the unlabelled tetraploid G_2M cell population As the IdUrd labelled cells move through the S-phase, the mean DNA content of the population will approach the DNA content of the G_2M population. IdUrd labelled cells which have divided and appeared in the (labelled) diploid G_1 region were excluded from the calculation of mean DNA content (figure 1). The movement of the IdUrd labelled S-phase cells relative to the position of G_0/G_1 and G_2M is expressed as relative movement (RM) and is calculated as follows:

$$RM = (F_{IdUrd} - F_{G0/G1}) / (F_{G2M} - F_{G0/G1})$$

F_{IdUrd} = mean DNA content of the IdUrd labelled cell (IdUrd labelled G_0G_1 cells were excluded)

F_{G0G1} = mean DNA content of the unlabelled diploid G_0G_1 population (will also contain the differentiated cell population)

F_{G2M} = mean DNA content of the G_2M cells

RM will increase in time from RM=0 5 at time=0 (IdUrd labelled cells half-way between the G_0G_1 cells and the G_2M cells) to RM=1 when all IdUrd labelled cells have reached tetraploid DNA content (IdUrd labelled cells which have divided are excluded) The time needed for labelled cells to reach tetraploid DNA content is equal to T_s T_s is calculated from one single sample using the formula:

$$T_s = 0\,5 / (RM - 0.5) \times \Delta t$$

RM = relative movement and Δt = time between pulse label and sampling

3. Results

3.1. Estimation of the Growth Fraction

Staining with MAb Pab601 can be used as an estimate of the germinative compartment in both normal and hyperproliferative epidermis Although the exact specificity of Pab601 is unknown, it is believed to react with either keratin 5 or keratin 14, or both (see chapter II). MAb Ki-67 recognizes an proliferation-associated nuclear antigen (see chapter IV) However, Ki-67 also bound to a cytoplasmic antigen, present predominantly in germinative cells derived from normal skin and skin from patients with abnormal keratinization (ichthyosis, Darier's disease, BFCP) Quantification of nuclear Ki-67 staining in section from

these tissues was very difficult. Cytoplasmic staining of Ki-67 was less in psoriatic lesional epidermis, and showed a patchy pattern in lesions from patients with atopic eczema. The growth fraction (GF) is defined here as the ratio of Ki-67-positive nuclei over Pab601-positive cells.

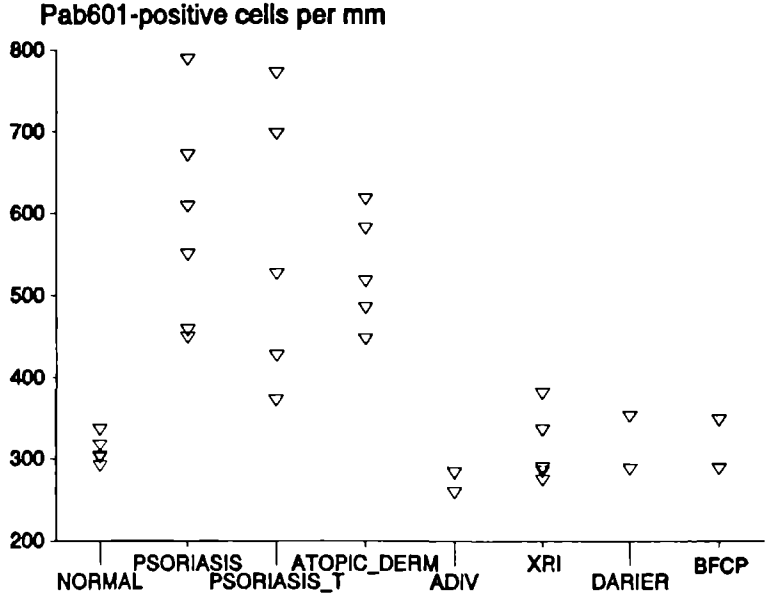


Figure 2 Number of Pab601-positive cells per mm in normal and diseased epidermis. ADIV = autosomal dominant ichthyosis vulgaris; XRI = X-linked recessive ichthyosis.

Our results obtained with the antibody Pab601 are given in figure 2. Values are given as number of Pab601-positive cells per mm length of section. The number of Pab601-positive cells was increased significantly in treated ($p<0.05$) and untreated ($p<0.05$) psoriatic epidermis compared to normal epidermis. There was no significant difference between treated and untreated psoriatic epidermis. Pab601 values were also increased in epidermis from patients with atopic dermatitis. Epidermis of the other skin disorders showed Pab601 staining in the normal range. In sections with increased numbers of Pab601-positive cells, staining was usually not restricted to the basal layer only, but the lower 2-3 layers of the epidermis were positive.

Within the diseases we studied, the absolute numbers of Ki-67-positive

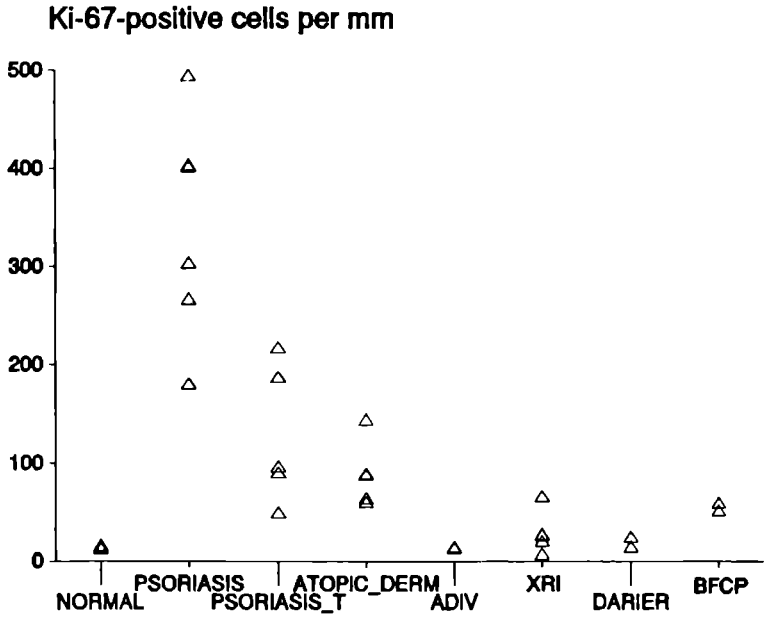


Figure 3 Ki-67-positive nuclei per mm.

nuclei in psoriasis and atopic dermatitis were much higher compared to normal skin (figure 3). Although, in treated psoriasis the number of Ki-67-positive nuclei was significantly decreased compared to untreated patients ($p<0.05$), this number was still higher than in normal epidermis. Whereas in psoriasis increased numbers of Ki-67-positive cells could be observed throughout the germinative layers of the lesion, in cryostat sections of lesions from patients with atopic dermatitis Ki-67 staining was confined to certain areas, most often those areas underlying parakeratotic stratum corneum. In skin disorders characterized predominantly by abnormal keratinization (i.e. ichthyosis and Darier's disease) Ki-67 values seemed much smaller.

Mean values of absolute numbers of positive cells for Pab601 and Ki-67, and the ratio Ki-67 over Pab601 are given in table I. The Ki-67/Pab601 ratio estimates the fraction of cycling cells in the germinative compartment independent of the total cell number in the epidermis. This ratio is defined here as the GF. In this study, the GF varied from 0.04 (normal epidermis) to 0.73 (untreated psoriatic epidermis). This means that in this particular case more than

Table I Mean values for Pab601, Ki-67 and the Ki-67/Pab601 ratio. Values are showed ± S.D. and with the number of observations in parenthesis.

DIAGNOSIS	Pab601/mm	Ki-67/mm	Ki-67/Pab601 ratio
normal epidermis	312 ± 17 (5)	13 ± 2 (5)	0.04 ± 0.01 (5)
psoriasis	589 ± 130 (6)	340 ± 113 (6)	0.57 ± 0.12 (6)
psoriasis (treated)	560 ± 172 (5)	127 ± 71 (5)	0.23 ± 0.12 (5)
atopic dermatitis	531 ± 69 (5)	88 ± 34 (5)	0.17 ± 0.06 (5)
ADIV	273 ± 17 (2)	13 ± 1 (2)	0.05 ± 0.01 (2)
XRI	315 ± 45 (5)	28 ± 22 (5)	0.09 ± 0.05 (5)
Darier's disease	322 ± 46 (2)	18 ± 7 (2)	0.06 ± 0.03 (2)
BFCP	320 ± 42 (2)	54 ± 6 (2)	0.17 ± 0.01 (2)

70% of the germinative cells were cycling. There was a significant difference between the GF in treated and untreated psoriatic epidermis ($p<0.05$). Furthermore GF in atopic dermatitis and BFCP were also increased compared to normal epidermis.

3.2. Double Immunoenzymatic Staining Method

Cycling cells and cells in S-phase of the cell cycle were visualized simultaneously, using MAb Ki-67 and a monoclonal anti-Brdurd antibody. Ki-67 was tested in the range 1:4 to 1:50. Higher Ki-67 concentrations resulted in more nuclear signal. However, with dilutions in the range 1:4 to 1:10 the background signal (in particular cytoplasmic labelling of basal cells) interfered with the nuclear staining. Every batch of peroxidase-conjugated second antibody as well as alkaline phosphatase-conjugated second antibody was tested for optimum concentration. Although small variations did exist, dilutions of 1:25 and 1:10, respectively, were generally used. After hydrolysis in 4 N HCl, anti-BrdUrd antibody was added and visualized using alkaline phosphatase-conjugated goat anti-mouse IgG. The primary idea of hydrolysis was denaturating DNA, but it was also destroying all the antibodies present in the cryostat section without interfering with the insoluble peroxidase product. Control experiments in which the second labelling step with anti-BrdUrd and the alkaline phosphatase conjugate were omitted revealed similar Ki-67 results.

Experiments in which we first denatured DNA, labelled with anti-BrdUrd, and Ki-67 was used as the second MAb failed, presumably due to destruction of the nuclear antigen for Ki-67.

Both antibodies recognize antigens located in the cell nucleus. Cell nuclei binding anti-BrdUrd (i.e. having incorporated BrdUrd) are in S-phase of the cell cycle (see chapter III). These BrdUrd-positive cells should also bind Ki-67. We found that nuclei staining blue (alkaline phosphatase) had a brown precipitate (peroxidase) underneath as well. A grossly increased number of BrdUrd-positive nuclei as well as Ki-67-positive nuclei could be observed in psoriatic lesional epidermis compared to normal epidermis.

Evaluation of Ki-67 binding in normal epidermis was sometimes difficult due to staining of the cell cytoplasm in addition to the nuclear binding of the MAb. In contrast to normal skin, Ki-67 staining of cycling cells in hyperproliferative epidermis such as psoriasis gave much less problems regarding non-specific cytoplasmic staining. Mean values of numbers of BrdUrd-positive

Table II BrdUrd-positive and Ki-67-positive cells per mm section length and the calculated LI for the various groups of specimens. All values are means ± S.D.

SPECIMEN	BrdUrd +	Ki-67 +	LI	n
normal epidermis (N)	8.6 ± 2.6	24.7 ± 5.8	0.35 ± 0.03	7
psoriasis uninvolved (PU)	6.3 ± 1.6	19.7 ± 4.4	0.32 ± 0.02	4
psoriasis involved (treated) (PL_T)	62.1 ± 22.1	191.2 ± 57.4	0.32 ± 0.03	8
psoriasis involved (untreated) (PL_U)	76.1 ± 24.9	196.9 ± 59.3	0.39 ± 0.08	7
atopic dermatitis (AD)	48.8 ± 20.1	135.3 ± 72.2	0.37 ± 0.05	3

and Ki-67-positive cells per mm length are given in table II. Increased numbers for BrdUrd-positive cells as well as Ki-67-positive cells were found in lesional epidermis of psoriasis and atopic dermatitis.

The ratio of the number of S-phase cells and the number of cycling cells for each condition is given in Table II (col.4). Similar mean values were obser-

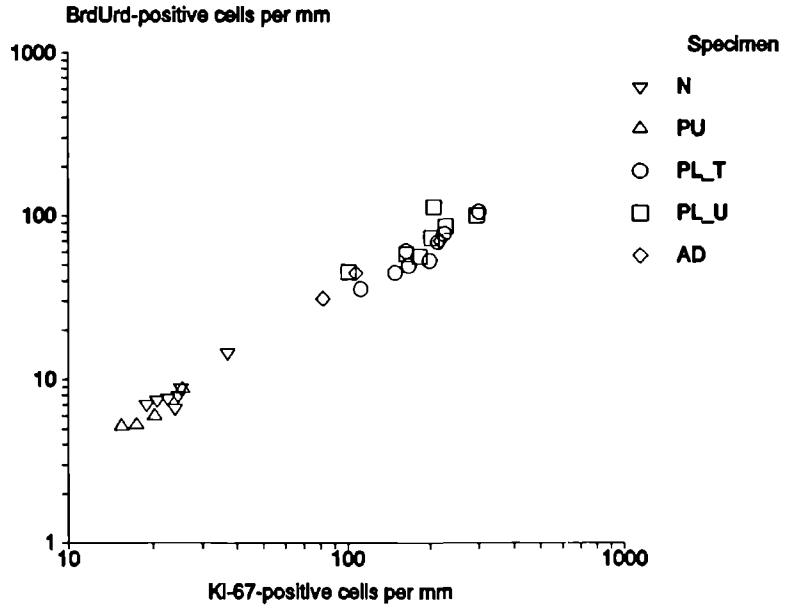


Figure 4 Correlation between numbers of BrdUrd-positive and numbers of Ki-67-positive cells in individual biopsies. Abbreviations: see table II.

ved for normal as well as hyperproliferative skin. A scattergram of the data for individual biopsies is illustrated in figure 4; no major differences in ratios of BrdUrd positive cells/Ki-67 positive cells (N_s/N_{cy}) were found (correlation coefficient = 0.961, $p<0.01$). The theoretical implications of this ratio for the calculation of cell cycle times is discussed in detail in section 4.

Because data to support the specificity of Ki-67 in human epidermis are limited, experiments were carried out to test the suitability of the antibody for staining proliferating epidermal keratinocytes (see also chapter IV). Firstly, the majority of human keratinocytes cultured on 3T3 feeder cells in the presence of serum express Ki-67 antigen. After 48 h serum starvation about 30% of the cells became Ki-67-negative. Secondly, 40 h after a standardized trauma of the skin (sellotape-stripping of the stratum corneum) in order to induce hyperproliferation, an induction was observed of Ki-67-positive cells in the lower layers of the epidermis.

3.3. Flow Cytometric Analysis Using Monoclonal Antibodies Against Intermediate Filaments

Flow cytometric analysis of four different MAb against intermediate filament-type proteins, in addition to measurement of relative DNA content, was performed on cell suspensions derived from lesional and clinically uninvolved skin of psoriatic patients and from skin of healthy controls. MAb K_s8.12, reacting with keratins 13 and 16 was used as a marker for hyperproliferation. Pab601 recognizes the basal cell layer(s) of human epidermis. Keratin 10 expression as a marker of keratinization was quantified with RKSE60 and the anti-vimentin MAb MVI was used as a marker for non-keratinocytes.

Table III summarizes the MAb staining results. Results are expressed as the percentage FITC-positive cells of all cells measured. Relative pool sizes of RKSE60-positive cells are significantly decreased in psoriatic lesional skin ($p<0.001$) compared to normal skin and compared to distant psoriatic

Table III Values of percentage MAb-positive cells in normal skin, psoriatic lesional skin, and distant uninvolved skin of psoriatic patients*.

SPECIMEN	RKSE60 (n)	Pab601 (n)	K_s8.12 (n)	MVI (n)
normal	57.2 ± 3.5 (17)	36.4 ± 4.5 (5)	1.8 ± 1.0 (6)	7.6 ± 3.0 (5)
psoriatic uninvolved	53.9 ± 6.1 (6)	ND	1.0 ± 0.2 (2)	7.0 ± 3.5 (5)
psoriatic lesion	46.6 ± 8.3 (9)	ND	40.4 ± 19.5 (11)	3.3 ± 2.1 (6)

*Mean ± S.D.
ND, not done.

uninvolved skin ($p<0.05$). In the case of vimentin-positive cells a significant decrease ($p<0.05$) was only observed in cells obtained from lesional skin compared to normal skin. The difference between normal and psoriatic skin with respect to K_s8.12 binding is striking ($p<0.001$). In normal skin and distant uninvolved skin from psoriatic patients K_s8.12-positive cells are found in very low amounts. In lesional skin of psoriatic patients, however, the percentage

K_s8.12-positive cells can vary from 10-60%. Percentage cells in S-phase and G_2M-phase of the cell cycle in psoriatic lesional skin is significantly increased compared to normal skin ($p<0.001$, chapter II, p. 39).

3.4. Incorporation of IdUrd *in vivo*.

Simultaneous measurement of the incorporated IdUrd and relative DNA content by FCM yields detailed information regarding cell kinetics. In particular, N_s and T_s can be obtained in this way. Parallel samples were stained also with the anti-keratin 10 MAb RKSE60, to quantify the suprabasal differentiated

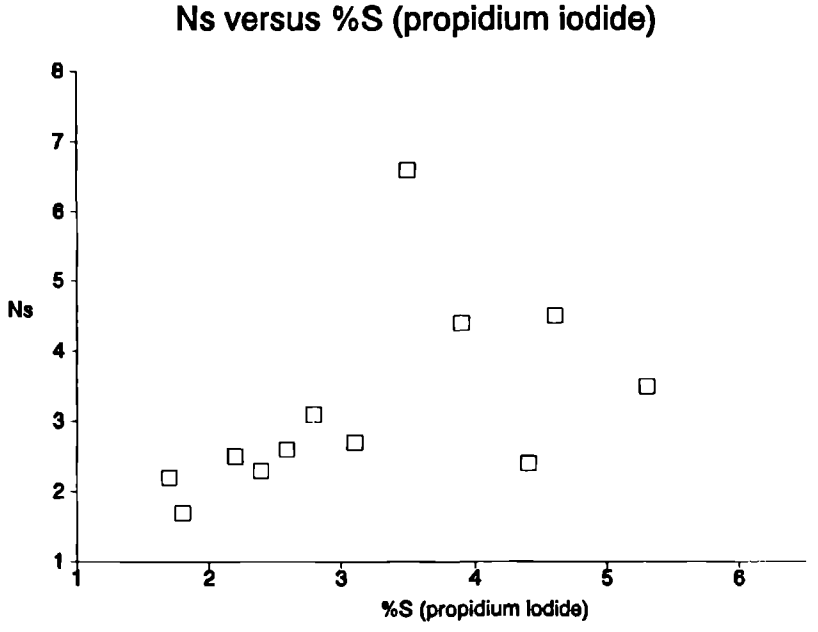


Figure 5 Scattergram of percentage cells in S-phase (x-axis) against N_S (y-axis).

compartment. Although skin specimens were more than 0.2 mm thick, preparation of cell suspensions was carried out using standard trypsinization conditions. However, they were cut into 1 mm pieces before trypsinization using a pair of scissors. N_s was found to be 3.5% ± 0.4 (mean ± S.E.M., n=13). The N_s was calculated as the sum of IdUrd-labelled cells with SG_2M DNA content and 0.5 x IdUrd-labelled cells with diploid DNA content (divided cells). Mean percentage cells with S-phase DNA content as calculated from DNA histograms was 3.5 ± 0.4 (n=13). In figure 5 N_s of the 13 individual

biopsies is plotted against %cells in S-phase. The average duration of the S-phase (T_S) was estimated to be 9.7 h ± 0.6. No correlation was found between T_S and N_S (figure 6).

The mean percentage RKSE60-positive cells in this group of normal controls, as a quantitative measure of the differentiated compartment was 59.6 ± 4.6 (n=12), and proved to be similar to earlier findings. There seems to be an inverse relationship between percentage RKSE60-positive cells and N_S (figure 7).

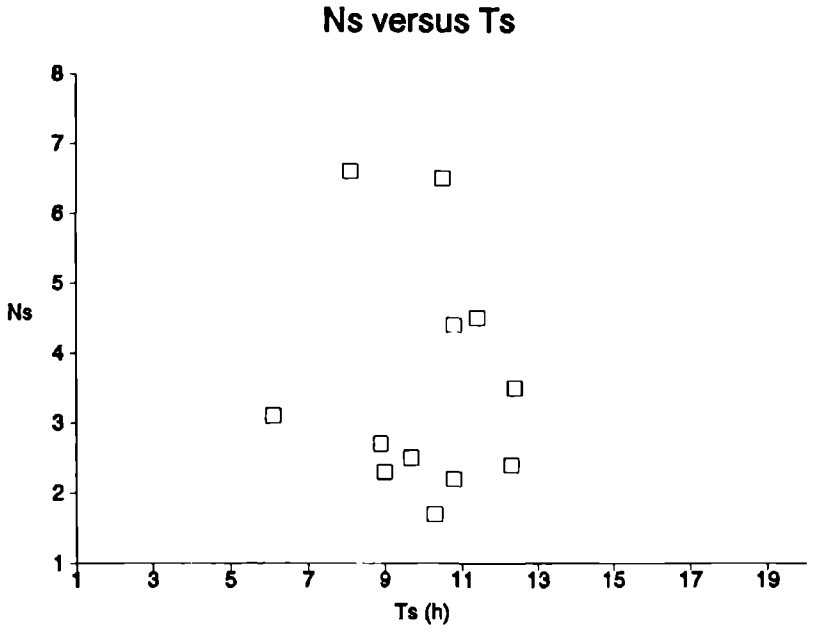


Figure 6 Scattergram of duration of the S-phase (T_S) on the x-axis and N_S on the y-axis.

4. Discussion

In this chapter we have applied some of our recently developed techniques to normal and diseased skin. Combining data obtained by phenotyping with MAb directed against intermediate filament-type proteins, MAb Ki-67, and data derived from cell kinetic studies, we have found that the population of germinative cells as measured by the MAb Pab601, was grossly increased in psoriatic lesional epidermis compared to normal epidermis. Secondly, we have

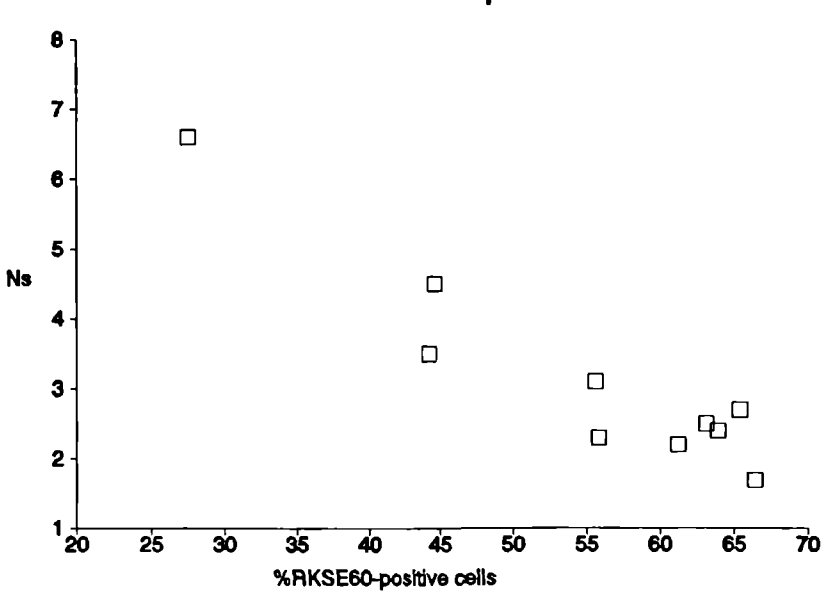


Figure 7 Relationship between percentage RKSE60-positive cells (x-axis) and N_S (y-axis).

shown a relative reduction of cells expressing keratin 10, whereas the number of K_s8.12-positive cells was grossly increased. Furthermore, we have shown additional evidence that the dramatic overproduction of keratinocytes found in psoriatic skin, characterized macroscopically by thickening of the epidermis and abnormal scaling, can be explained by an increased number of cycling cells rather than a reduced cell cycle time.

Because of their relatively poor solubility compared to most other proteins, intermediate filament-type proteins can be identified easily in normal and psoriatic epidermis by means of SDS gel-electrophoresis. Basically, two keratin abnormalities have been observed in psoriatic epidermis using this technique. Firstly, a reduction and sometimes even absence of a high molecular weight keratin (keratin 1)[19,20] and secondly, the induction of two hyperproliferation-specific low molecular weight keratins (keratins 6 and 16)[21,22]. However, information about specific location, which cells and how many cells express a specific keratin is lost.

After preparation of epidermal cell suspensions from normal, psoriatic

uninvolved and psoriatic lesional skin, quantification of RKSE60 binding using a fluorescence microscope revealed a relative reduction of the number of keratin 10-positive cells in psoriasis[23]. To the best of our knowledge no differences have been published between normal and psoriatic epidermis with respect to expression of keratins specific for the basal layer (keratins 5 and 14) using SDS gel electrophoresis. Here it is shown immunohistochemically that Pab601 staining in psoriatic lesional epidermis is not restricted to the basal cell layer but that the lowest two or three cell layers are Pab601-positive. The boundary between positive and negative cells is not clear. Using K_c8.12, keratin 16 expression has been shown in the suprabasal layers of hyperproliferative skin[24], including the psoriatic lesion[25]. In normal skin no suprabasal staining could be shown, however, a minor patchy staining of the basal layer was seen.

Important in the studies with anti-keratin antibodies was that immunohistochemistry and FCM were combined. This means that epidermal characteristics were assessed on a per cell basis and many cells were analyzed rapidly when FCM was used, whereas morphological characteristics and topographical relations were obtained by immunohistochemistry.

The results obtained with Ki-67 and anti-BrdUrd suggest the existence of a population of resting (G_0) cells in normal and hyperproliferative epidermis. In hyperproliferative epidermis, however, the majority of germinative cells are cycling, whereas in normal epidermis only a small fraction is cycling. The MAb Ki-67 used in this study to label all cells in S, G_2, M and the majority of cells in G_1, but not G_0 and differentiated cells[26], stains only a small proportion of all germinative cells in normal skin. In hyperproliferative skin disorders the number of Ki-67-positive cells increases dramatically.

Two methods are used in the literature to determine the number of keratinocytes in S-phase of the cell cycle: autoradiography and cytometry[27]. Using autoradiography after incorporation of a radioactive DNA precursor ([^{3}H] thymidine or [^{14}C] thymidine) into DNA, cells actively synthesizing DNA can be visualized. With cytometric methods the DNA content of individual cells can be measured using a quantitative DNA stain. In this way the fraction of cells in S-phase can also be determined. For our studies we have chosen a recently developed method using a MAb to detect actively DNA synthesizing

cells (chapter III). BrdUrd and IdUrd, both pyrimidine analogues of thymidine can be identified in the nucleus after their incorporation into DNA by means of an anti-BrdUrd (or anti-IdUrd) MAb. This method gives results similar to autoradiography[28,29], but has the advantage that straight immunohistochemical methods can be used to quantify S-phase cell numbers.

Total numbers of Ki-67-positive and BrdUrd-positive cells in hyperproliferative skin disorders are both grossly increased, as shown in Table II. Absolute numbers using per mm length of cryostat section should be interpreted with caution because of technical aspects, for instance tissue orientation when sections are cut[30] and because of differences in tissue architecture when psoriatic lesional skin and normal skin are compared. However, combining both Ki-67 and anti-BrdUrd labelling in a sequential double staining a true independent LI can be measured, according to the following equation:

$$LI = \frac{N_s}{N_c}$$

where N_s is the number of S-phase cells and N_c the number of cycling cells. Table II (col.4) shows the LI for normal skin, psoriatic lesional skin, psoriatic uninvolved skin and lesional skin of patients with atopic dermatitis. Obviously, the values of LI, using this approach, are much higher than those reported in the literature, because we have used the cycling cell population as the reference, whereas others have used an estimate of the total germinative compartment[31,32]. In contrast to these latter studies we show that LI in hyperproliferative skin is not different to that found in normal skin. This remarkable finding is best illustrated by plotting the number of BrdUrd-positive cells per unit length against the number of Ki-67-positive cells per unit length for each individual (figure 4).

Calculation of absolute cell cycle times depends on the assumption that cells are randomly distributed through the various phases of the cycle. Although this assumption has been questioned[33], it has been used by various investigators in this field (for example by Bauer[27]). It follows that:

$$\frac{N_s}{N_c} = \frac{T_s}{T_c}$$

where T_S is the duration of S-phase and T_C is the cell cycle time. The ratio of N_S over N_C was determined from the sequential double staining, and T_s for normal epidermis was calculated from the *in vivo* IdUrd labelling experiments, being 9.7 h ± 0.6. Our results were similar to results from the literature[27], which gives T_s relatively constant for normal epidermis as well as psoriatic lesion (varying from 6.5 h to 16 h, averaging about 10 h). In the studies of normal epidermis so far, only small numbers of cells (either radioactive-labelled S-phase cells or M-cells) were counted on slides to determine T_s. Thus the precision of the estimates obtained for normal epidermis was poor. None of the data in the literature were obtained with the BrdUrd (idUrd)-technique. The cell cycle time of dividing keratinocytes in human skin can be calculated in this way to be 28 h. This figure is compatible with that of 26 h found by direct observation of keratinocytes in culture (chapter VII), with the value of about 25 h found by Rijzewijk et al[34] and also with 39 h using the stripped skin model[35].

This study demonstrates that an immunohistochemical double staining technique can be an useful tool in studying cell cycle kinetics in epithelial tissues in general, and in epidermis in particular. Using ratios of numbers of BrdUrd-positive cells over numbers of Ki-67-positive cells methodological and technical problems with respect to cell counting were omitted. The technique is reproducible and accurate despite the many steps that are needed; labelling with the antibodies must be sequential because of the denaturation step required for anti-BrdUrd binding.

With respect to the cell kinetic results we state firstly that, although evidence is accumulating, in particular for blood cells and also for other cell types that Ki-67 is a reliable marker for cycling cells, further research concerning its specificity in epidermis is urgently needed. The appearance of Ki-67-positive and Ki-67-negative cells in several layers in the basal region of psoriatic lesional skin, suggests an increased germinative compartment, as we have showed using MAb against intermediate filament-proteins. Because total epidermis is also much thicker, there is a relative increase of the germinative compartment from 30% to 50%[27]. However, the absolute numbers of Ki-67-positive and BrdUrd-positive cells have increased in psoriatic lesional skin 8- to 10-fold (table II).

In conclusion, using MAb against intermediate filament-type proteins, the sequential double immunoenzymatic staining procedure, and application of *in vivo* IdUrd labelling, we show that there seems no major difference between normal and hyperproliferative skin with respect to cell cycle time our data instead suggest an increased GF. Differences reported in the literature could be the result of overestimation of the GF in normal epidermis. Furthermore, the inverse relationship found between percentage RKSE60-positive cells and N_s *in vivo* supports the idea that proliferation and differentiation are integrated processes. Table IV summarizes cell kinetic data of normal epidermis and epidermis from psoriatic patients. The production rate can be calculated by dividing N_c by T_c. Assuming that the number of keratinocytes per surface area is 4 times higher in a psoriatic lesion, this means that the production rate in psoriatic lesional epidermis is at least 15 times higher when compared with normal epidermis.

Table IV Summary of cell kinetic data on normal and psoriatic lesional epidermis.

	normal	psoriatic lesion
N_{diff}	60%	45%
N_{germ}	40%	55%
N_C/N_{germ}	<0.1	0.3-1
T_S	10 h	10 h
T_C	30 h	30 h

We think that further research concerning increased cell proliferation and its significance for the pathogenesis of psoriasis and other hyperproliferative skin disorders should be focused onto the mechanisms involved in controlling the recruitment of resting (G_0) cells and the roles of other epidermal subpopulations besides the germinative subpopulation (differentiated cells, Langerhans cells, infiltrate cells).

5. References

1. Van Scott EJ, Ekel T: Kinetics of hyperplasia in psoriasis. Arch Dermatol 88: 373-380, 1963

2. Weinstein GD, van Scott EJ: Autoradiographic analysis of turnover times of normal and psoriatic epidermis. J Invest Dermatol 45: 257-262, 1965

3 Weinstein GD, Frost P Abnormal cell proliferation in psoriasis J Invest Dermatol 50 254 259, 1968

4 Voorhees JJ, Duell EA, Bass LJ, Powell JA, Harrell ER Decreased cyclic AMP in the epidermis of lesions of psoriasis Arch Dermatol 105 695-701, 1972

5 Voorhees JJ, Duell EA Psoriasis as a possible defect of the adenyl cyclase-cyclic AMP cascade Arch Dermatol 104 352-358, 1971

6 Roelfzema H, Bergers M, van Erp PEJ, Gommans JM, Mier PD Studies on the plasma membrane of normal and psoriatic keratinocytes 3 Uptake of labelled sugars and their incorporation into glycoconjugates Brit J Dermatol 104 635-640, 1981

7. Roelfzema H, Bergers M, van Erp PEJ, Gommans JM, Mier PD Studies on the plasma membrane of normal and psoriatic keratinocytes 4 Characterization of glycoconjugates Brit J Dermatol 105 509 516, 1981

8 Roelfzema H, van Erp PEJ Glycoprotein composition of psoriatic epidermis in relation to growth control J Invest Dermatol 80 20-23, 1983

9 Mann PR, Williams RH, Gray GM Distribution of glycoproteins containing fucose in normal and psoriatic keratinocytes Brit J Dermatol 102 649 657, 1980

10 Forster S, Ilderton E, Summerly R, Yardley HJ The level of phospholipase A_2 activity is raised in the uninvolved epidermis of psoriasis Brit J Dermatol 108 103 105, 1983

11 Van de Kerkhof PCM, van Erp PEJ Calmodulin levels are grossly elevated in the psoriatic lesion Brit J Dermatol 108 217-218, 1983

12 Tucker WFG, MacNeil S, Bleehan SS, Tomlinson S Biologically active calmodulin levels are elevated in both involved and uninvolved epidermis in psoriasis J Invest 82 298 299, 1984

13 Gottlieb AB, Chang CK, Posnett DN, Fanelli B, Tam JP Detection of transforming growth factor (alpha) in normal, malignant, and hyperproliferative human keratinocytes J Exp Med 167 670 675, 1988

14 Elder JT, Fisher GJ, Lindquist PB, Bennett GL, Pittelkow GL, Coffey RJ, Ellingsworth L, Derynck R, Voorhees JJ Overexpression of transforming growth factor (alpha) in psoriatic epidermis Science 243 811-814, 1989

15 Nanney LB, Stoscheck CM, Magid M, King LE Altered epidermal growth factor binding and receptor distribution in psoriasis J Invest Dermatol 86 260 265 1986

16 Gentleman S, Martensen TM, Digiovanna JJ, Chader GJ Protein tyrosine kinase and protein phosphotyrosine phosphatase in normal and psoriatic skin Biochem Biophys Acta 798 53 59, 1984

17 Bauer FW, Boezeman JBM Flow cytometric methods in human skin with respect to cell cycle kinetics in Psoriasis cell proliferation Eds Wright NA, Camplejohn RS Edinburgh, Churchill Livingstone, 1983 pp 104 116

18 Begg AC, McNally NJ Shrieve DC, Kärcher H A method to measure the duration of DNA synthesis and the potential doubling time from a single sample Cytometry 6 620-626, 1985

19 Thaler MP, Fukuyama K, Inone N, Cram DL, Epstein WL· Two tris urea mercaptoethanol extractable polypeptides found uniquely in scales of patients with psoriasis. J Invest Dermatol 70 38-41, 1978

20 Skerrow D, Hunter I Protein modifications during the keratinization of normal and psoriatic human epidermis Biochem biophys Acta 537. 474-484, 1978

21 McGuire J, Osber M, Lightfoot L· Two keratins MW 50,000 and 56,000 are synthesized by psoriatic epidermis Brit J Dermatol 111 (Supplement 27) 27-37, 1984

22 Weiss RA, Eichner R, Sun T-T: Monoclonal antibody analysis of keratin expression in epidermal diseases a 48- and 56-kdalton keratin as molecular markers for hyperproliferative keratinocytes J Cell Biol 98. 1397-1406, 1984

23 Bauer FW, Boezeman JBM, van Engelen L, de Grood RM, Ramaekers FCS Monoclonal antibodies for epidermal population analysis J Invest Dermatol 87 72-75, 1986

24 Huszar M, Gigi-Leitner O, Moll R, Franke WW, Geiger B Monoclonal antibodies to various acidic (type I) cytokeratins of stratified epithelia Selective markers for stratification and squamous cell carcinomas Differentiation 31 141-153, 1986

25 De Mare S, van Erp PEJ, van de Kerkhof PCM Epidermal hyperproliferation assessed by the monoclonal antibody K_s8 12 on frozen sections J Invest Dermatol 92 130-131, 1989

26 Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki 67. J Immunol 133 1710-1715, 1984

27 Bauer FW Cell kinetics in Textbook of psoriasis Eds Mier PD, van de Kerkhof PCM Edinburgh, Churchill Livingstone, 1986, pp 100-112

28 Gunduz N The use of FITC-conjugated monoclonal antibodies for determination of S-phase cells with fluorescence microscopy Cytometry 6 597-601, 1985

29 Langer E-M, Röttgers HR, Schliermann MG, Meier E-M, Miltenburger HG, Schumann J, Göhde W Cycling S phase cells in animal and spontaneous tumours I comparison of the BrdUrd and ^{3}H-thymidine techniques and flow cytometry for the estimation of S-phase frequency Acta Radiol Oncol 24 545-548, 1985

30 Leigh IM, Pulford KA, Ramaekers FCS, Lane EB Psoriasis maintenance of an intact monolayer basal cell differentiation compartment in spite of hyperproliferation Brit J Dermatol 113 53-64, 1985

31 Weinstein GD, Ross P, McCullough JL, Colton A Proliferative defects in psoriasis in Psoriasis cell proliferation Eds Wright NA, Camplejohn RS Edinburgh, Churchill Living stone, 1983, pp 189 208

32 Weinstein GD, McCullough JL, Ross PA Cell kinetic basis for pathophysiology of psoriasis J Invest Dermatol 85 579 583, 1985

33 Wright N, Alison A Kinetic parameters in stratified squamous epithelia in The biology of epithelial cell populations Oxford, Clarendon Press, 1984, pp 250-286

34. Rijzewijk JJ, Boezeman JBM, Bauer FW: Synchronized growth in human epidermis following tape-stripping: its implication for cell kinetic studies. Cell Tissue Kinet 21:227-229, 1988

35. Boezeman JBM, Bauer FW, de Grood RM: Flow cytometric analysis of the recruitment of G0 cells in human epidermis in vivo following tape stripping. Cell Tissue Kinet 20:99-107, 1987

CHAPTER VII. APPLICATION OF CYTOMETRIC METHODS TO STUDY EPIDERMAL GROWTH AND DIFFERENTIATION IN VITRO

This chapter was based on the following publication

Piet EJ van Erp, Jan BM Boezeman, Gijs J de Jongh, Joost Schalkwijk, Epidermal growth and differentiation in vitro studied with flow cytometric and immunohistochemical techniques. Manuscript in preparation.

CHAPTER VII. APPLICATION OF CYTOMETRIC METHODS TO STUDY EPIDERMAL GROWTH AND DIFFERENTIATION IN VITRO

1. Introduction 121
2. Methods & Materials 123
   2.1. Passage and Culture of Swiss Mouse 3T3 Fibroblasts (123)
   2.2. Keratinocyte Culture and Tissue Preparation (123)
   2.3. Maintenance of Cultured Keratinocytes in Suspension (123)
   2.4. IdUrd Labelling (124)
   2.5. Immunohistochemistry (124)
   2.6. Denaturation and Staining for Flow Cytometry (124)
   2.7. Flow Cytometry (126)
3. Results 126
   3.1. Immunohistochemistry (126)
   3.2. Bivariate IdUrd/DNA Analysis after a Pulse Label (129)
   3.3. Quantitative Flow Cytometric Analysis (130)
4. Discussion 133
5. References 137

1. Introduction

Keratinocyte growth and differentiation *in vivo* can be modulated by the local environment, for example, directly by mesenchymal-epithelial interactions, or by cytokines produced by other cell types. However, despite this dependence on environmental influences, it seems that the tendency of the keratinocyte to stratify and differentiate is retained *in vitro*, even in the absence of instructive information from the connective tissue stroma[1] and despite the fact that basal cells of different epithelia have diverged irreversibly from each other during development, resulting in different programs of differentiation[2,3]. These findings suggest that a culture system for human epidermal keratinocytes can be a good model for cellular growth and differentiation, especially to study the effect of environmental influences.

Early methods for culturing keratinocytes were hampered by problems with mechanical dissociation of epidermal tissue, or the need for very high inoculation densities. In addition, the lifespan of normal keratinocytes was limited, and fibroblast overgrowth continued to be a major problem. Neither glass nor plastic proved to be an ideal substrate for epidermal cells in culture. Collagen coating of plastic petri dishes improved the culture technique by enhancing attachment and growth[4,5]. Régnier et al[6] developed a technique for the cultivation of keratinocytes on de-epidermized human skin. On top of the dermis the basement membrane was functionally intact and as such a substrate for the keratinocytes. The authors claimed that differentiation under their conditions was enhanced, and that several markers of keratinization were found. Maintenance of the cultures at the culture medium-air interface induced even more complete or complex differentiation. However, a major problem with the techniques described above was that cell production was limited. This rapid, exponential growth of keratinocytes, from single cells to macroscopic colonies, was achieved by using a feeder technique[7]. Human keratinocytes obtained by trypsinization of newborn foreskin, and later also adult skin, were co-cultivated with gamma-irradiated 3T3 cells (Swiss mouse fibroblasts). Terminal differentiation of the cells was incomplete. Some degree of differentiation occurred when the cultures reached confluence. Keratinocytes derived from foreskin of newborn humans could be serially cultivated in the presence of 3T3 cells through a total of 50 cell generations. It was believed that the number of generations could be substantially extended if epidermal growth factor (EGF) was added to the culture medium[8].

In this study our aim was to analyze growth and differentiation of exponentially growing and confluent cultures of human keratinocytes, using immunohistochemical and flow cytometric techniques described in the previous chapters. Secondly, we wanted to investigate the possibility of using cultured keratinocytes as an *in vitro* model for recruitment of quiescent cells. Furthermore, an extended *in vitro* model was evaluated in which trypsinized keratinocytes derived from confluent cultures were maintained in suspension by continuous rotation. Green and others have shown that prevention of keratinocyte adhesion to a substratum results in the induction of terminal differentiation[9,10,11].

2. Methods & Materials

2.1. Passage and Culture of Swiss Mouse 3T3 Fibroblasts

The cells were cultured in 20 cm^2 petri dishes. Cells were passaged 2 times weekly, on Monday and Friday. The culture medium was Dulbecco's modified Eagle's medium (DMEM, Gibco, Breda, The Netherlands), containing 10% newborn calf serum (Flow Laboratories, Irvine, Scotland), 100 U/ml penicillin (Gibco, Breda, The Netherlands), and 100 μg/ml streptomycin. On passage the medium was removed, and the cells were washed once with PBS. Trypsinization was carried out using a solution containing 0.25% trypsin (Difco 1:250, Gibco, Breda, The Netherlands), 0.02% EDTA, and 0.1% glucose. The incubation time was 30 seconds. The trypsin solution was removed totally, and the incubation was continued for another 15 min at 37°C in a CO_2-incubator. Then 3.3 ml culture medium was added and the suspension was finally distributed over 3 new petri dishes, which contained 5 ml culture medium each.

2.2. Keratinocyte Culture and Tissue Preparation

Human keratinocytes derived from adult skin, were cultured on feeder cells[7]. The epidermal cells were seeded on lethally irradiated (3000 Rad in 3 min) Swiss mouse 3T3 fibroblasts in DMEM/F12 3:1 v/v (Flow Laboratories, Irvine, Scotland) supplemented with 0.4 μg/ml hydrocortisone (Collaborative Research Inc., Lexington, MA, USA), 10^{-6}M isoproterenol (Sigma, St.Louis, MO, USA), 100 U/ml penicillin plus 100 μg/ml streptomycin (Gibco, Breda, The Netherlands), 6% v/v fetal bovine serum (Seralab, Nistelrode, The Netherlands) and, starting at day three after seeding, with 10 ng/ml EGF (Collaborative Research Inc., Lexington, MA, USA). Cells were grown at 37°C, 95% relative humidity, and 7.5% CO_2 in air. EDTA treated, trypsinized keratinocytes from the first 1-2 passages were used in the experiments.

2.3. Maintenance of Cultured Keratinocytes in Suspension

Confluent keratinocyte cultures were trypsinized using a solution containing 0.25% trypsin (Difco 1:250, Gibco, Breda, The Netherlands), 0.02% EDTA, and 0.1% glucose. Trypsinized cells were washed once and resuspended in DMEM/F12 medium (3:1 v/v) as described in section 2.2. EGF was not added. The cell suspension derived from one petri dish (about 10^7 cells) was collected in one 10 ml plastic tube and culture medium was added till the tube

was filled completely. Tubes were placed in a rotating device as drawn schematically in figure 1, which completed one rotation in 4 min. Preliminary experiments showed that cells kept vital up to 5 days. However, from day 3 cells started aggregating and could not be used for flow cytometrical analysis. A standard maintenance period of 48 h was chosen, and during the last 30 h IdUrd-labelling was performed.

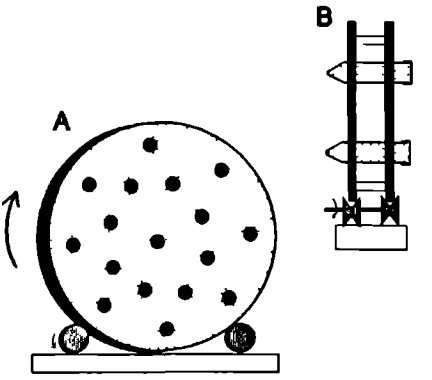


Figure 1 A: Front view of rotating device for suspension maintenance of human keratinocytes. B: Insert shows side-view.

2.4. IdUrd Labelling

Cell cultures and cells maintained in suspension (10^6 cells/ml) were labelled 30 h *in vitro* with IdUrd (Sigma, St.Louis, USA) in order to measure the growth fraction (GF). Pulse labelling of cell cultures was performed using a 1 h pulse and reincubation in IdUrd-free medium. The labelled cells were sampled in time up to 30 h after the pulse. The final IdUrd concentration for both experimental conditions was 10 μM. Cells were harvested, washed in ice-cold PBS fixed in ice-cold 70% ethanol and stored at -20°C until use.

2.5. Immunohistochemistry

An indirect immunoperoxidase staining was performed, both on cells grown on coverslips and cells adherent to poly-l-lysine-coated microscope slides, using primary and secondary antibodies as listed in table I. Details of the staining protocol can be found in chapter II. In the case of staining with DAKO-BrdUrd (Dakopatts, Copenhagen, Denmark), hydrolysis was performed as described in chapter III. AEC (Sigma, St.Louis, USA) together with H_2O_2 were used as enzyme substrates.

2.6. Denaturation and Staining for Flow Cytometry

Between 0.5-1.0x10^6 ethanol-fixed cells were used for staining with a variety of antibodies (table II). Cells were washed once with PBS. In the case of staining with DAKO-BrdUrd a hydrolysis step was included using the pepsin/HCl protocol described in chapter III. Hydrolysis was terminated with

Table I Overview of primary and secondary antibodies used with the immunoperoxidase technique.

ANTIBODY	SPECIFICITY	SOURCE	CODE	DILUTION
K40 (rabbit)	spectrum of keratins	dept. Pathology[1]		1:10
MVI (mouse)	vimentin	Eurodiagnostics	MVI	...
RKSE60 (mouse)	keratin 10	dept. Pathology		1:10
K_S8.12 (mouse)	keratin 13 and 16	Sigma	C-7034	1:40
RCK102 (mouse)	keratin 5 and 8	dept. Pathology		1:20
DAKO-BrdUrd (mouse)	BrdUrd and IdUrd incorporated in DNA	Dakopatts	M-744	1:20
Ki-67 (mouse)	human proliferation-associated nuclear antigen	Dakopatts	M-722	1:20
α-involucrin (rabbit)	involucrin	BTI[2]	BT-601	1:8
α-filaggrin (mouse)	filaggrin	BTI	BT-576	1:500
RAM-PO[3]		Dakopatts	P-161	1:50
SWAR-PO[4]		Dakopatts	P-217	1:50

[1]University of Nijmegen
[2]Biomedical Technologies Inc, Stoughton, USA
[3]Peroxidase-conjugated rabbit immunoglobulins (Ig) to mouse Ig
[4]Peroxidase-conjugated swine Ig to rabbit Ig

excess 0.1 M $Na_2B_4O_7$. After a minimum of 2 washes with PBS, denatured/protein-digested cells were pelleted. These cells, and in the case of the other antibodies, the cells after the first PBS wash step, were incubated with appropriate dilutions of primary and secondary antibody. In the case of labelling with Ki-67-FITC the incubation step with secondary antibody was omitted. After incubation with the second antibody in all other cases, the cells were washed and resuspended in 400 μl PBS containing 20 μg/ml PI (Calbiochem, San Diego, CA, USA) and incubated for 15 min with 50 μl 1% w/v RNAse (Sigma, St. Louis, MO, USA). Prior to flow cytometric analysis cell suspensions were filtered through gauze (mesh 50 μm).

Table II Overview of primary and secondary antibodies used for flow cytometric analysis (for specificity of the antibodies see table I).

ANTIBODY	SOURCE	CODE	DILUTION
K40 (rabbit)	dept. Pathology[1]		1:10
K_S8.12 (mouse)	Sigma	C-7034	1:20
RKSE60 (mouse)	dept. Pathology		1:10
HN-IU (mouse) (anti-IdUrd)	dept. Haematology[1]		1:10
DAKO-BrdUrd (mouse)	Dakopatts	M-744	1:20
Ki-67-FITC (mouse)	Dakopatts	F-788	1:20
Ki-67 (mouse)	Dakopatts	M-722	1:5
α-involucrin (rabbit)	BTI[2]	BT-601	1:8
α-filaggrin (mouse)	BTI	BT-576	1:500
RAM-FITC[3]	Dakopatts	F-313	1:25
SWAR-FITC[4]	Dakopatts	F-205	1:25

[1]University of Nijmegen
[2]Biomedical Technologies Inc, Stoughton, USA
[3]FITC-conjugated rabbit immunoglobulins (Ig) to mouse Ig
[4]FITC-conjugated swine Ig to rabbit Ig

2.7. Flow Cytometry

Cells stained with FITC and PI were measured with a flow cytometer 50H (Ortho Instruments, Westwood, CA, USA) equipped as described in chapter II.

3. Results

3.1. Immunohistochemistry

Figure 2 shows how human keratinocytes were grown *in vitro* in the presence of a feeder of irradiated 3T3 cells. Keratinocytes of the first or second passage were used, and these keratinocytes formed visual colonies within 2-3 days after inoculation. Confluent epithelia were grown from these colonies in 9-14 days. In general, cultured keratinocytes grew as a monolayer. However, in the centre of the colonies from which the confluent epithelium was

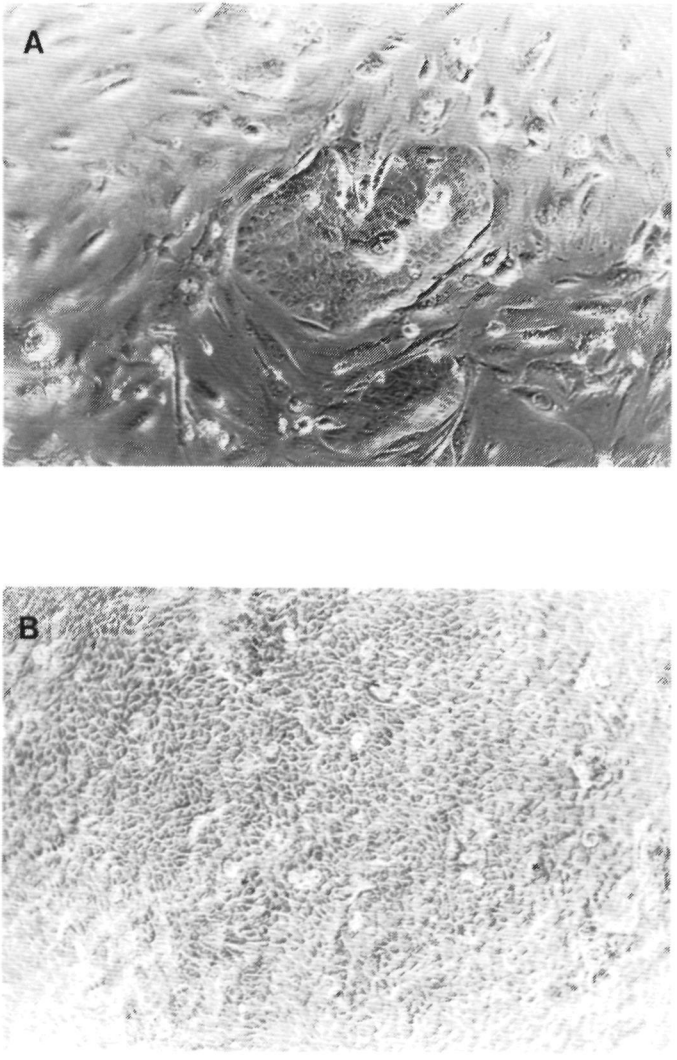


Figure 2 Phase-contrast photographs of exponentially growing keratinocytes (A) and keratinocytes grown to confluency (B) *in vitro*.

formed, the cells underwent terminal differentiation. Cells from those areas were continuously discharged as squames.

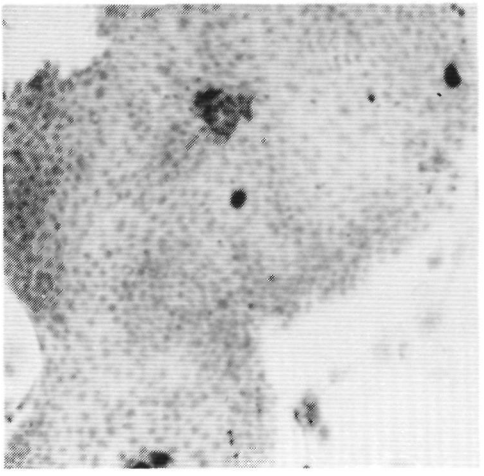


Figure 3 Immunoperoxidase staining of coverslips using the monoclonal antibody RKSE60. Very few cells stain positive in the centre of the colonies.

Coverslips containing either exponentially growing cells (i.e. less then 30% of the petri dish contained epidermal cells) or confluent cells were studied. The 3T3 fibroblasts used as feeder cells were negative for all antibodies tested.

All exponentially growing keratinocytes and all cells in confluent cultures stained positively with the epithelial cell-specific polyclonal antibody K40, and with the basal cell-specific monoclonal antibody RCK102 (see figure 5A in chapter II, p. 40). Sporadically human fibroblasts could be detected in the cultures by intense staining with the anti-vimentin antibody MVI. A minority of the keratinocytes also expressed vimentin. However staining of these cells was less intense. Only very few cells expressed keratin 10 as visualized by RKSE60 binding. In general the desquamating keratinocytes in the centre of a colony were positive for keratin 10 (figure 3). The number of keratin 10-positive cells increased slightly

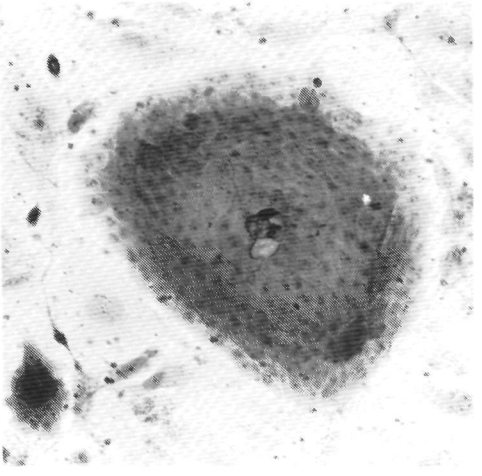


Figure 4 Immunoperoxidase staining of coverslip using the monoclonal antibody K_S8.12. Cells at the periphery of the colonies are K_S8.12-negative.

when cultures became confluent.

A significant difference between subconfluent and confluent cultures was found in the staining patterns with K_s8.12. While under confluent conditions all keratinocytes were K_s8.12-positive, under subconfluent conditions the cells at the periphery of the colonies were K_s8.12-negative (figure 4).

With respect to the proliferation-specific antibodies Ki-67 and DAKO-BrdUrd a dramatic reduction of positively stained nuclei was observed when cultures were reaching confluency. Reduction of the number of Ki-67-positive nuclei was accompanied by a slightly increased cytoplasmic staining.

3.2. Bivariate IdUrd/DNA Analysis after a Pulse Label

Figure 5 shows bivariate IdUrd/DNA distributions measured for exponentially growing keratinocytes after a 1 h exposure to 10 μM IdUrd. Cells were harvested at 0, 3, 6, 16, 24, and 30 h after the pulse label. Each panel in figure 5 is a two-parameter cytogram relating the green fluorescence (IdUrd content vertically) of each cell to its red fluorescence (DNA content horizontal-

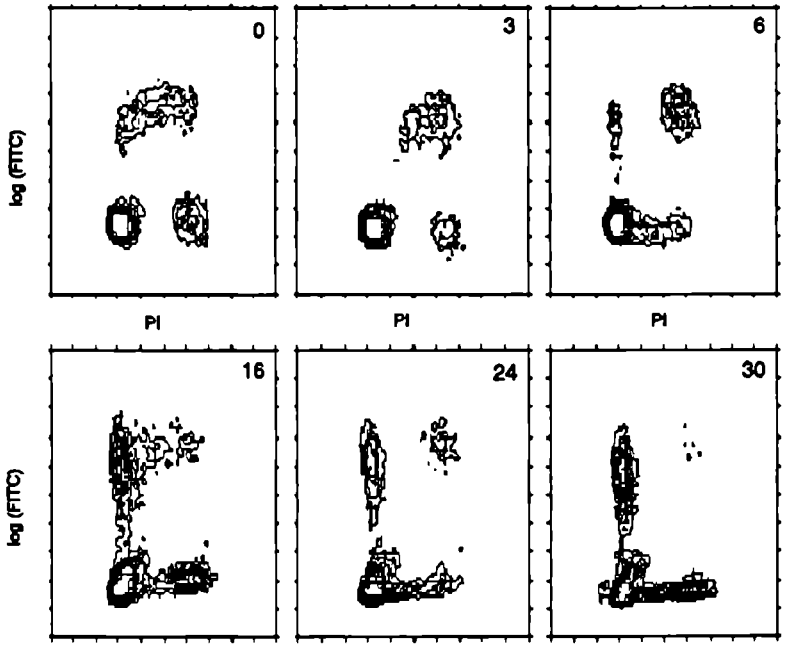


Figure 5 Bivariate IdUrd/DNA distributions with PI (DNA content) on the x-axis, and FITC (DAKO-BrdUrd) on the y-axis. Time intervals (h) are indicated in the individual plots.

ly). At least 10,000 cells were analyzed for each cytogram. At time=0, immediately after pulse labelling with IdUrd, the cells which stained with DAKO-BrdUrd lay between two populations without green fluorescence, G_1 cells on the left and G_2 cells on the right. At later times, the IdUrd-positive population moved progressively to the right (t=3 h), through S-phase into G_2. Once these cells had completed mitosis, IdUrd-positive cells occurred in G_1-phase (t=6 h). By 16 h, some of the cells that had originally been in S-phase had moved through G_2, M, and G_1 and reappeared in S. For the exponentially growing keratinocytes in figure 5, a cell cycle time can be estimated of 20 h. In 3 additional experiments using exponentially growing keratinocytes from different volunteers, similar cell cycle times were found ranging between 20-30 h. When keratinocytes were used derived from confluent cultures, again similar cell cycle times were observed.

3.3. Quantitative Flow Cytometric Analysis

Flow cytometric analysis using proliferation-specific and differentiation-specific antibodies in combination with measurement of relative DNA content was performed on single cell suspensions derived from exponentially growing and confluent human keratinocyte cultures, and keratinocytes maintained in suspension. Early experiments were carried out using keratinocytes derived from neonatal human foreskin. However, using this source, proliferating activity of the cultures stayed high, even under confluent culture conditions. Percentage cells in S and G_2M-phase of the cell cycle was found to be 28.4±6.0 (7) (mean ± S.D. and the number of cultures in parenthesis). When adult skin-derived keratinocyte cultures were analysed, a value of 10.8±2.4 (12) was found (see also table III). Restimulation of these confluent cultures with fresh serum did not result in a measurable recruitment of quiescent cells.

The polyclonal antibody K40 was used in order to exclude the possibility of contamination with non-keratinocytes (human fibroblasts, 3T3 cells). In all experiments described in this study, the percentages keratinocytes in the cell suspensions were more than 95%. When keratinocyte cultures were grown to confluency in a 60 mm petri dish, they contained about 10 times as many cells as the subconfluent cultures. Morphological differences of the cells derived from subconfluent and confluent cultures were minimal. However, when keratinocytes derived from confluent cultures were maintained in suspension

their sizes increased, and most of the cells lost their nuclei after 2-5 days. After 2-4 days the cells became permeable to trypan blue. Maintenance of cells in suspension for periods of more then 2 days was not practical, because cells started to aggregate and flow cytometric analysis became impossible.

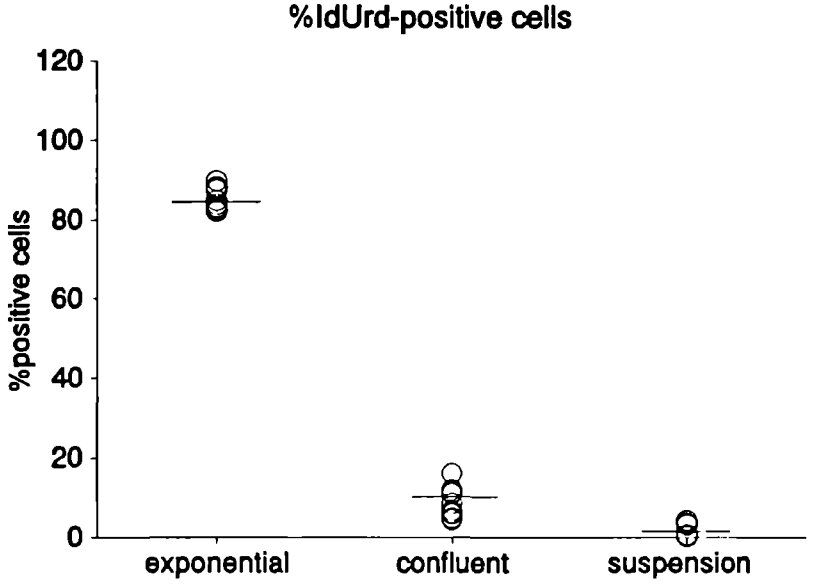


Figure 6 Growth fractions measured by continuous IdUrd incorporation. IdUrd-positive cells were visualized using DAKO-BrdUrd.

Aliquots of cells were labelled with the antibodies listed in table II. Cells were simultaneously stained with the quantitative DNA fluorochrome PI. The DNA values from this double labelling technique were identical to those obtained with single-parameter measurements, indicating that no artifacts were introduced by measuring more than one parameter at once and an increased number of incubation and wash steps. DNA values calculated from bivariate IdUrd/DNA cytograms were also identical to results obtained with the other antibodies, despite the additional hydrolysis and proteolytic digestion step. When human keratinocytes were growing exponentially about 35% of the cells were in S and G_2M-phase of the cell cycle. This value decreased significantly when the cultures reached confluence. Under suspension conditions SG_2M-values increased again, however measurements were hindered by the fact that cells

started to lose their nuclei.

Flow cytometric quantification of the growth fraction for individual samples, as measured by continuous (30 h) labelling with IdUrd and detection of the IdUrd-positive cells by the MAb DAKO-BrdUrd, is shown in figure 6. In subconfluent cultures growth fractions were over 80%, whereas confluent cultures entered a steady state with about 10% cycling cells. When these cells were kept in suspension they stopped DNA-synthesis completely. An alternative technique to quantify the growth fraction is nuclear Ki-67 binding. The results

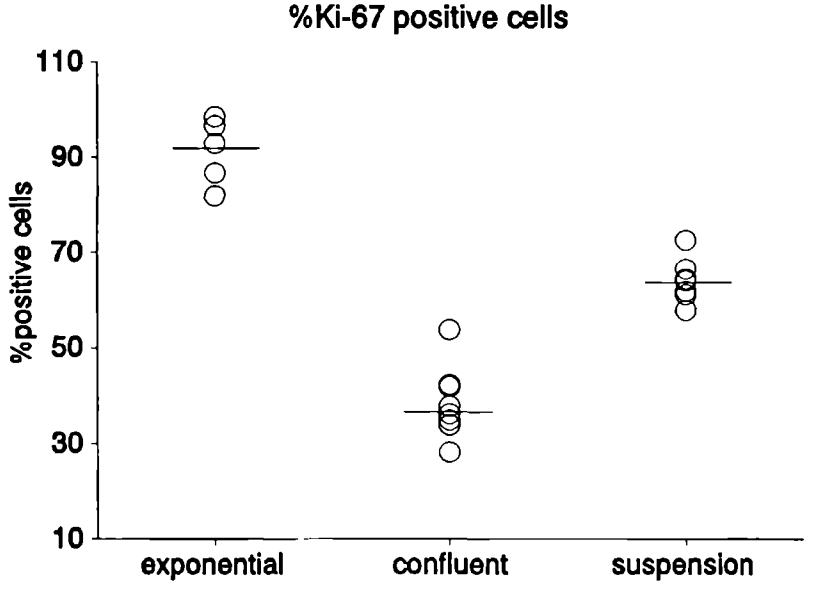


Figure 7 Quantification of the growth fraction using the MAb Ki-67.

are shown in figure 7. Surprisingly, Ki-67 values were very high in suspension maintenance. Light microscopical assessment revealed an additional cytoplasmic staining pattern. In cell suspensions derived from confluent cultures, percentage Ki-67-positive cells was lower than in cell suspensions derived from subconfluent cultures. However, a percentage of 38% was much to high, when compared to the DAKO-BrdUrd value.

Values found for K_s8.12 and RKSE60 were in line with the immunohistochemical findings. The number of cells binding K_s8.12 was higher in con-

fluent cultures compared to subconfluent cultures. This value decreased slightly when the cells were maintained in suspension. Keratin 10 expression as visualized by RKSE60 binding, was very low in human keratinocyte cultures, although a small increase seemed evident under suspension conditions.

Involucrin expression was induced when keratinocytes were grown to confluence, and in keratinocytes maintained in suspension for two days up to half of the population was stained with anti-involucrin (figure 8).

Results with anti-filaggrin as a differentiation marker were similar to values obtained with RKSE60.

Table III summarizes the flow cytometric data by giving mean values and standard deviations for all proliferation and differentiation markers, subdivided in three experimental culture conditions.

4. Discussion

Epidermopoiesis, the process of controlled continuous production of keratinocytes, therefore involving proliferation and differentiation, has been subject to many investigations in the past (for review[12]). Cultured human keratinocytes have been used as a model system to study epidermal growth and differentiation using immunohistochemical and flow cytometric techniques. Study of this process *in vivo* is complex, due to the heterogeneity of the epidermal cells, and the interference by systemic factors and infiltrate cells. For this reason, *in vitro* studies, in which no systemic influences and other cell types are interfering, can contribute to a better understanding of the complex regulating mechanisms. However, it has been repeatedly shown that, using the standard culture technique of Rheinwald and Green[7], epidermal cells can be induced to differentiate only to a certain extent[13]. Morphologically, epidermal cells growing as a monolayer are different from the epidermis *in vivo*. By these morphologic criteria, the keratinocyte stratification *in vitro* does not proceed further than what is observed in the lower layers of the epidermis, namely mainly the basal and suprabasal layers. This was confirmed by the fact that in both subconfluent and confluent cultures the majority of the keratinocytes were stained positively with the basal cell-specific antibody RCK102, and by the lack of staining with the differentiation-specific antibody RKSE60. Furthermore, only few cells were positive for an anti-filaggrin antibody, and

less then 10% of the cells stained with anti-involucrin. However, the phenotype of the keratinocytes *in vitro* was found to differ from normal basal cells *in vivo* with respect to vimentin and keratin 13 and/or keratin 16 expression.

In cultured keratinocytes vimentin was found in more than 20% of the cells. This is in agreement with the results of Van Muijen et al[14] who showed that 40-70% of the basal cells were vimentin-positive. Others, however, did not find vimentin expression in cultured keratinocytes[15]. A possible explanation for

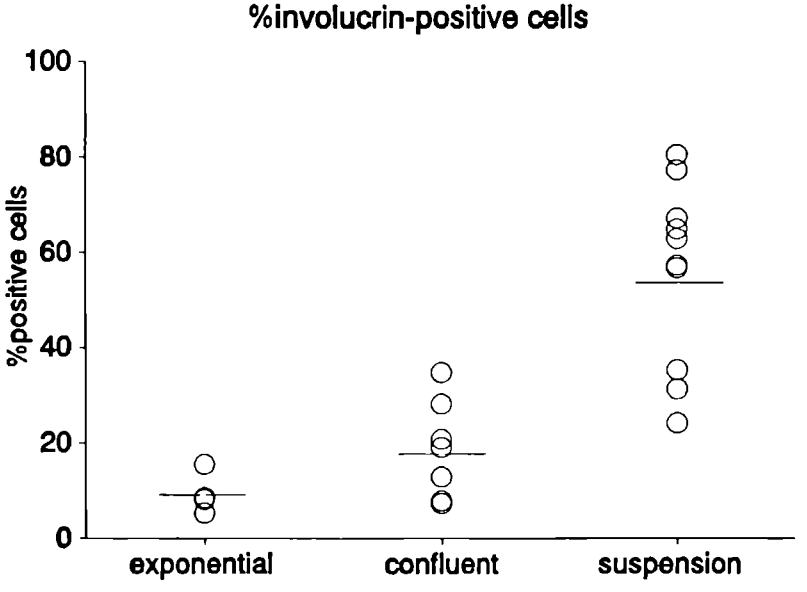


Figure 8 Binding of anti-involucrin in human cultured keratinocytes subdivided in three experimental culture conditions.

this discrepancy may be that keratinocytes used for culturing were obtained from different sites or that the culture conditions were different. For instance, Rheinwald et al[16] reported that keratinocytes grown under low-Ca^{++} conditions did not express vimentin, whereas keratinocytes cultured without 3T3 feeder cells were found to be vimentin-positive.

Although normal basal cells *in vivo* do not react with the antibody $K_s8.12$[17], the majority of keratinocytes in vitro are positively stained with the antibody. $K_s8.12$-positive cells are primarily located in the upper layers of

hyperproliferative epidermis[17]. In subconfluent cultures we found that K_s8.12-binding was restricted to the central area of growing colonies. When these cultures reached confluency and entered a steady state where less than 10% of the cells were dividing, all keratinocytes were found positive for K_s8.12. These immunohistochemical data were confirmed by flow cytometric data showing that the number of K_s8.12-positive cells was increasing from 82.7% in subconfluent cultures to 98.4% in confluent cultures. Van Muijen et al[14] showed that in confluent cultures only about 30% of the superficial cells were positive for keratin 13. This means that only a minority of the K_s8.12-binding can be due to expression of keratin 13, and that the majority of the keratinocytes express keratin 16, even under steady state conditions with only little proliferating activity. This suggests that keratin 16 expression is not only a marker of hyperproliferation as proposed by Weiss et al[18] and others, but also could be a marker of abnormal differentiation.

Table III *Mean values (± S.D.) for percentage of cells in S and G2M-phase of the cell cycle, and percentage MAb-positive cells in cultured keratinocytes.*

	SG2M	DAKO-BrdUrd	Ki-67	K_s8.12	RKSE60	α-Involucrin	α-Filaggrin
Exponential	35.9 ± 2.6 (12)	85.6 ± 2.8 (11)	91.3 ± 6.9 (5)	82.7 ± 7.1 (4)	7.9 ± 1.1 (4)	9.4 ± 4.3 (4)	13.4 ± 1.8 (2)
Confluent	10.8 ± 2.4 (12)	9.5 ± 4.0 (12)	38.1 ± 7.3 (9)	98.4 ± 0.7 (5)	1.9 ± 0.8 (5)	18.7 ± 10.3 (7)	6.5 ± 3.3 (5)
Suspension	22.1 ± 2.9 (5)	2.8 ± 1.5 (7)	64.0 ± 4.7 (7)	87.6 ± 5.5 (6)	11.0 ± 9.2 (6)	53.6 ± 19.5 (10)	15.4 ± 6.8 (8)

Measurement of proliferative activity was performed using three different approaches. As a result of the double labelling techniques as described in detail in chapter II, relative DNA content is measured simultaneously with antibody binding. Additionally we have measured the GF using two alternative methods, namely bivariate IdUrd/DNA analysis and Ki-67/DNA analysis. The GF is defined here as the fraction of cells that are actively cycling at a given

time[11]. For GF measurements using the IdUrd/DNA technique, a 30 h exposure period with IdUrd was chosen, because of a cell cycle time for cultured human keratinocytes of 25-35 h[19,20,21]. The same value was also estimated from the pulse label experiments described in section 3.2. This means that over a 30 h labelling period all proliferating cells had the opportunity to incorporate IdUrd in their DNA. In principle, the IdUrd/DNA technique is a powerful technique for detailed cell cycle kinetics and for measuring GF. However, use of IdUrd or BrdUrd *in vivo* in humans is limited due to ethical considerations involved in administering a precursor of DNA and the requirement for multiple biopsies if cell cycle measurements are to be made. Additionally, in the case of continuous labelling the result obtained is an integrated measure of a preceding period (in this case 30 h), assuming that no major changes occur during that period. Alternatively, the monoclonal antibody Ki-67 can be used for estimations of the GF. Ki-67 recognizes a human proliferation-associated nuclear antigen[22] and could be measured simultaneously with relative DNA content using a standard indirect and direct immunofluorescent technique (chapter IV). Remarkably, in confluent cultures the Ki-67 GF was invariably higher than the IdUrd GF. A possible explanation for this finding could be that cells withdrawn from the cell cycle under confluent culture conditions retain the antigen recognized by Ki-67 for a considerable period of time.

Several successful attempts have been made to induce more complete differentiation *in vitro*. Nearly physiological *in vitro* conditions were created when keratinocytes were cultured on an air-liquid interface using de-epidermized dermis as a substrate[23]. When mechanical pressure was exerted on keratinocyte monolayers periodically, they locally grew up to several layers and the number of horny cells increased[24]. However, these techniques are complex, time-consuming, and preparation of single cells from the cultures is laborious. This makes these culture techniques in principle less suitable to flow cytometric analysis. In 1977 Green reported that when growing colonies of epidermal keratinocytes were disaggregated and the cells were suspended in medium containing methyl cellulose, they could not multiply, but within a few days started to differentiate synchronously[9]. We have modified and simplified this technique and kept keratinocytes derived from confluent cultures in

suspension using a rotating device (figure 1). Differentiation was indeed induced by preventing the keratinocytes from adhering to the plastic surface of the tubes. In agreement with results reported by Green using methyl cellulose-containing medium, cell size was increasing and cells started to lose their nuclei[9]. The number of involucrin-positive cells increased to more than 50%. Simultaneously, cells stopped multiplying. Only IdUrd GF could be used for quantification of cell cycle withdrawal, because measurement of relative DNA content became difficult due to nuclear disaggregation, and Ki-67 measurements were hindered by cytoplasmic staining as seen in normal epidermis *in vivo*[25]. Together with small increases in the number of filaggrin-positive and RKSE60-positive cells, and a slight reduction of K_s8.12-positive cells, these results suggest a differentiation program closer to normal terminal differentiation *in vivo* than with standard confluent culture conditions.

The results presented in this chapter demonstrate that cultured human keratinocytes can be used as a model system to study epidermal growth and differentiation. Using a combination of immunohistochemical and novel flow cytometrical techniques it is possible to obtain a detailed description of these vital processes. An improvement and extension of the standard culture method according to Rheinwald and Green[7] was achieved by maintenance in suspension of keratinocytes derived from confluent cultures. Under those conditions, cells were not capable of dividing, started to lose their nuclei, and the expression of differentiation-related proteins such as involucrin and filaggrin was induced, suggesting that these cells changed towards a differentiated phenotype resembling normal differentiated cells *in vivo*. Keratin 16 expression occurred under all conditions, suggesting that it is a marker for abnormal differentiation rather than for hyperproliferation.

5. References

1. Flaxman BA, Lutzner MA, van Scott EJ: Cell maturation and tissue organization in epithelial outgrowths from skin and buccal mucosa *in vitro*. J Invest Dermatol 49:322-332, 1967

2. Doran TI, Vidrich A, Sun T-T: Intrinsic and extrinsic regulation of the differentiation of skin, corneal and esophageal epithelial cells. Cell 22:17-25, 1980

3. Van Erp PEJ, Bergers M, de Grood RM, Koedam J, Rijntjes JM: Fucosyl glycopeptide profiles of keratinocytes from various epithelial tissues of the rabbit in relation to differentiation in vivo and in vitro. J Invest Dermatol 83:359-362, 1984

4 Karasek MA, Charlton ME Growth of postembryonic skin epithelial cells on collagen gels J Invest Dermatol 56 205-210, 1971

5 Murray JC, Stingl G, Kleinman HK, Martin GR, Katz SI Epidermal cells adhere preferentially to type IV (basement membrane) collagen J Cell Biol 80 197-202, 1979

6 Régnier M, Pruniéras M, Woodley D Growth and differentiation of adult human epidermal cells on dermal substrate Front Matrix Biol 9 4-35, 1981

7 Rheinwald JG, Green H Serial cultivation of strains of human epidermal keratinocytes the formation of keratinizing colonies from single cells Cell 6 331-334, 1975

8 Rheinwald JG, Green H Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes Nature 265 421-424, 1977

9 Green H Terminal differentiation of cultured human epidermal cells Cell 11 405-416, 1977

10 Rice RH, Green H Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope activation of the cross linking by calcium ions Cell 18 681 694, 1979

11 Adams JC, Watt FM Fibronectin inhibits the terminal differentiation of human keratinocytes Nature 340 307-309, 1989

12 Bauer FW Cell kinetics in Textbook of psoriasis Eds Mier PD, van de Kerkhof PCM Edinburgh, Churchill Livingstone, 1986 pp 100-112

13 Holbrook KA, Hennings H Phenotypic expression of epidermal cells in vitro a review J Invest Dermatol 81 11s 24s, 1983

14 Van Muijen GNP, Warnaar SO, Ponec M Differentiation related changes of cytokeratin expression in cultured keratinocytes and in fetal, newborn, and adult epidermis Exp Cell Res 171 331-345, 1987

15 Franke WW, Schmid E, Breitkreutz D, Lüder M, Boukamp P, Fusenig NE, Osborn M, Weber K Simultaneous expression of two different types of intermediate sized filaments in mouse keratinocytes proliferating *in vitro* Differentiation 14 35 50, 1979

16 Rheinwald JG, O Connell TM, Connell ND, Rybak SM, Allen-Hoffmann BL, LaRocca PJ, Wu Y-J, Rehwoldt SM Cancer cells The transformed phenotype Edited by Levine AJ, Van de Woude GF, Topp WC, Watson JD Cold Spring Harbor, Cold Spring Harbor Laboratory, Vol 1, 1984, pp 217

17 De Mare S, van Erp PEJ, van de Kerkhof PCM Epidermal hyperproliferation assessed by the monoclonal antibody K_S8 12 on frozen sections J Invest Dermatol 92 130 131 1989

18 Weiss RA, Eichner R, Sun T T Monoclonal antibody analysis of keratin expression in epidermal diseases a 48 and 56 kdalton keratin as molecular markers for hyperproliferative keratinocytes J Cell Biol 98 1397 1406 1984

19 Wille JJ, Pittelkow MR, Shipley GD Scott RE Integrated control of growth and differentiation of normal human prokeratinocytes cultured in serum free medium clonal analyses, growth kinetics, and cell cycle studies J Cell Physiol 121 31-44, 1984

20. Staiano-Coico L, Higgins PJ, Darzynkiewicz Z, Kimmel M, Gottlieb AB, Pagan-Charry I, Madden MR, Finkelstein JL, Hefton JM: Human keratinocyte culture - Identification and staging of epidermal cell subpopulations. J Clin Invest 77:396-404, 1986

21. Albers KM, Greif F, Woodrow Setzer R, Taichman LB: Cell-cycle withdrawal in cultured keratinocytes. Differentiation 34:236-240, 1987

22. Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H: Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 133:1710-1715, 1984

23. Régnier M, Schweizer J, Michel S, Bailly C, Pruniéras M: Expression of high molecular weight (67k) keratin in human keratinocytes cultured on dead de-epidermized dermis. Exp Cell Res 165:63-72, 1986

24. Görmar FE, Bernd A, Bereiter-Hahn J, Holzmann H: A new model of epidermal differentiation: Induction by mechanical stimulation. Arch Dermatol Res 282:22-32, 1990

25. Rijzewijk JJ, van Erp PEJ, Bauer FW: Two binding sites for Ki-67 related to quiescent and cycling cells in human epidermis. Acta Derm Venereol (Stockh) 69:512-515, 1989

CHAPTER VIII. FINAL CONSIDERATIONS

CHAPTER VIII FINAL CONSIDERATIONS

1. Cytometry as a tool to study epidermal growth and differentiation . 143
2. Heterogeneity in the germinative population of human epidermal keratinocytes 144
3. Expression of the differentiated phenotype 146
4. Regulation of human keratinocyte proliferation and differentiation .. 147
5 Future prospectives 149
6. References 149

1. Cytometry as a tool to study epidermal growth and differentiation

In cytometric methodology in general, and with techniques such as immunohistochemistry and flow cytometry in particular, the cell is considered as a functional unit It is important to realize that studies using exclusively these techniques can only give a limited amount of information with respect to the complex processes of growth and differentiation However, possibilities of the techniques are increasing Flow cytometric measurement of enzyme activities and signal transduction pathways demonstrate that the technique is capable of analyzing at the subcellular level Phenotyping, detection of specific gene products using monoclonal antibodies has become a standard procedure We have used the latter technique to give a more complete description of different compartments and cell types in the epidermis We have also shown that functional information can be obtained by measurement of pH_i and DNA synthesis In principle it is even possible to measure gene expression flow cytometrically by in situ hybridization[1].

The advantages and disadvantages of the individual techniques have been discussed in detail in the technique-specific chapters (chapter II - V) In general, the techniques have in common that they are novel, and allow qualitative as well as quantitative analysis of epidermal growth and differentiation beyond the possibilities presently available Applications are in fact not limited to epidermis, but can be used for studies on proliferation and differentiation of

any tissue, or *in vitro* cell system. However, it is important to realize that the strength of the methodology described in this thesis cannot be found in one particular technique, but that the techniques are complementary. One of the conclusions to draw is that one should not aim for the ultimate technique to measure either proliferation or differentiation, but one should use a panel of phenotypic and functional markers to evaluate the total process of epidermal growth and maturation. In this case it has been done from a cell biological point of view, but this approach will also stand when biochemical assays are used. Future search for new techniques will therefore not only be focused on whether they are better or can do more, but also to what extent they contribute to the panel of techniques already in use, and what information they add with respect to the processes studied.

2. Heterogeneity in the germinative population of human epidermal keratinocytes

In chapter I we have shown that the epidermis can be divided essentially into three compartments, i.e. germinative cells, differentiated cells, and stratum corneum cells. Whereas in the compartment of differentiated cells a morphological heterogeneity exists anyway, and also a functional heterogeneity seems very likely, the existence of heterogeneity in the germinative population of human epidermal keratinocytes is matter of much discussion in experimental dermatology.

The old concept of Weinstein[2] that all epidermal cells in the germinative compartment of human epidermis are actively proliferating with an equal cell cycle time for normal epidermal cells of 457 h, is still not totally repudiated by all dermatologists. However, in any renewal system, and therefore also in human epidermis, the cell production rate is not only determined by the cell cycle time, but also by the growth fraction. The growth fraction is then defined as the proportion of cells in any population which is actively engaged in the cell cycle. Thus a hyperproliferative state, such as psoriatic epidermis, can be due to a decrease in the cell cycle time or an increase in the growth fraction. In order to define the proliferative defect in psoriatic epidermis more precisely, we have to distinguish between the two. The measurements of Weinstein and co-workers were performed with the one major assumption that the growth

fraction is unity, and all cells are proliferating. Our point is that in normal human epidermis *in vivo* there are, most probably, a considerable number of germinative cells which are not proliferating. The first evidence for this statement originates from the work of Gelfant et al[3,4] using continuous [^{3}H]-thymidine labelling of normal epidermis *in vivo* in a limited number of human subjects. They concluded that a rough estimate of 50 to 60 percent of germinative epidermal cells may reside in the non-cycling state. More recently, additional evidence in favour of a resting G_0 population was brought about. Tape stripping of human skin invoked a cohort of synchronously-dividing cells, entering S-phase by 36 h after induction of the trauma[5]. This cohort was described as the recruitment of cells from a pre-existing G_0 compartment which consists of 76% of all proliferative cells. These authors calculated a cell cycle time of 39 h for normal human keratinocytes.

In this thesis we have showed that immunohistochemical staining with the MAb Ki-67 (chapter IV) labels only a minority of positive cells in the basal layer of normal human skin. Sections of psoriatic epidermis and epidermis 40 h after tapestripping showed large numbers of positive nuclei in the basal and suprabasal layers. Using a double labelling with Ki-67 and anti-BrdUrd we were able to demonstrate equal cell cycle times of 30 h for normal and psoriatic epidermis (chapter VI). The same value was found for cultured human keratinocytes (chapter VII). This result was in line with recent findings of others[6,7].

Some of these recent studies have indicated heterogeneity of the cycling population of keratinocytes and have inferred a more complex functional organization of the epidermal tissue. Although we were not able to show this heterogeneity up to now, we think that in vitro IdUrd-labelling studies with more time points could be a method to test the validity of these findings.

With respect to analysis of the germinative cell compartment we may conclude that the classic concept of a growth fraction of unity is no longer valid. The cell cycle time of normal proliferative human keratinocytes, proliferative epidermal cells derived from hyperproliferative tissue, and proliferating epidermal cells *in vitro*, is equal and in the order of 30-40 h. Differences in tissue turnover and tissue thickness are therefore most likely caused by changes in the growth fraction.

3. Expression of the differentiated phenotype

The maturation of keratinocytes to form cornified envelopes is accompanied by multiple biochemical changes, and without doubt the cytoskeleton plays an important role in this process. By far the most thoroughly studied components are the intermediate filament proteins. Keratinocytes can be

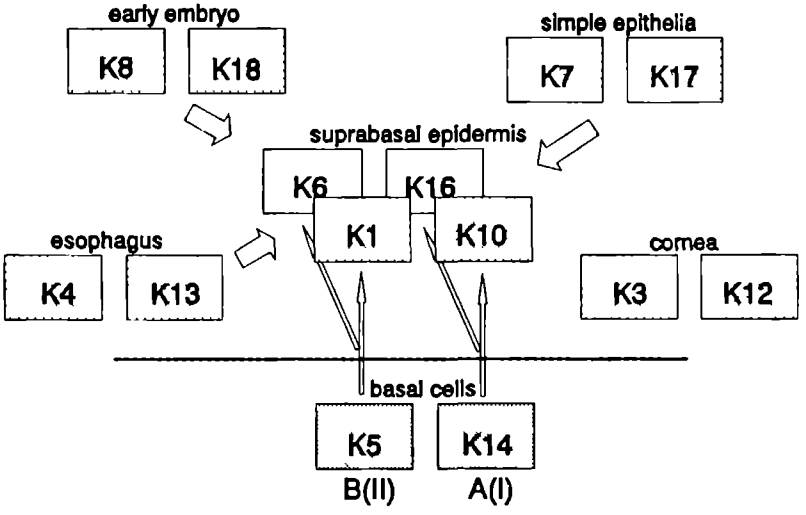


Figure 1 A given epithelium or epithelial cell can be characterized by a specific pattern of its keratin components.

recognized among all other cell types in skin, such as Langerhans cells, melanocytes, fibroblasts, and infiltrate cells, by the specific expression of keratin-type intermediate filament proteins. The keratinocytes themselves are functionally heterogeneous, ranging from germinative cells to those in the terminal, fully differentiated state. Keratins in all epithelial cells, including keratinocytes, tend to be expressed in pairs containing one acidic-type and one basic-type protein (figure 1). As shown in chapter II, the identification and quantification of epidermal cellular subpopulations was performed by using specific monoclonal keratin antibodies either on cryostat sections or in single cell suspensions using flow cytometry. Besides keratins other structural

proteins, in particular involucrin and filaggrin have been implicated in terminal differentiation. Filaggrin is a basic, histidine-rich protein and is synthesized by differentiating epithelial cells as a large precursor, known as profilaggrin[8]. Profilaggrin has been shown to constitute the major component of keratohyalin granules found in the stratum granulosum and therefore is considered a marker of late-stage differentiation. Involucrin is one of the substrates for the formation of cornified envelopes and is synthesized in normal epidermis in the stratum granulosum as well. This should imply that this protein is also a marker of late-stage differentiation. However, in pathological epidermis and in cultured keratinocytes this protein is synthesized much earlier[9]. Some cells in cuiture synthesized DNA and expressed involucrin simultaneously. This implies that the sequence of events leading to the formation of corneocytes is not the same for all situations, and presumably even not the same for all cells in a given situation. Reasonably, this differentiation process must be cautiously *regulated and parallel steps, such as induction of enzyme activity and substrate* biosynthesis, must be "synchronized" by the tissue. It is attractive to propose that the specific expression of keratins 6 and 16 could be a signal that this synchronization of the differentiation process is lost for some or all cells in a tissue, instead of being a hyperproliferative-specific pair of keratins (figure 1). If so, one must assume that in psoriatic epidermis keratin 16 expression as measured by K_s8.12 binding is also reflecting asynchronous differentiation. Whatever causes this abnormality remains to be elucidated. It is tempting to speculate that the overproduction of basal keratinocytes causes a disbalance in the sequence of differentiation steps; the overproduction itself being the result of a defect in the $G_0 \rightarrow G_1$ recruitment mechanism.

4. Regulation of human keratinocyte proliferation and differentiation

The epidermis is an excellent example of a dynamic tissue in which highly regulated mechanisms exist to control cell proliferation and differentiation. The ultimate and primary goal of all keratinocytes is to protect the organism against unwanted external influences. This means from a functional point of view that the process of cell production is in principle subordinated to the process of differentiation. However, experimental evidence, also from studies

in this thesis, is accumulating that the control of keratinocyte proliferation and differentiation is integrally regulated. Clonal analysis, growth kinetics, and cell cycle studies of normal human keratinocytes cultured in serum-free medium show that if keratinocyte proliferation is promoted, differentiation is inhibited and when differentiation is induced, proliferation is inhibited[10]. Also calcium modulation[11] and maintenance of cells in suspension (chapter VII) have been used to show that terminal differentiation could be initially stimulated at the expense of proliferation. An *in vivo* study using sellotape stripping, in which the outermost layer of cornified cells is removed by repeated application of adhesive tape, also suggests that proliferation and differentiation are integrated processes[12], and in chapter VI we show an inverse relationship between N_s and the percentage of RKSE60-positive cells.

A third process, the process of inflammation, although somewhat outside the scope of this thesis, should be mentioned. The existence of regulatory circuits between dermal and epidermal inflammatory infiltrate and the overlying epidermal keratinocytes has been implicated in the fine control of epidermal proliferation and turnover[13]. Evidence was presented that activated peripheral blood mononuclear cells produce factors that modify keratinocyte growth and differentiation, and it was suggested that these interactions may play a role in wound repair and skin pathology. In general, skin disorders with increased epidermal growth, such as psoriasis, are accompanied by cutaneous inflammation. Several *in vivo* models have been used by Van de Kerkhof and coworkers to investigate the interrelationship between epidermal growth and inflammation: the response of the skin to standardized injury, the response to epicutaneous application of leukotriene B_4 (LTB_4), and the various phases of the skin disorder psoriasis[14]. In these studies, a key role for the polymorphonuclear leukocyte (PMN) has been suggested. Alternatively, Valdimarsson et al propose that the process leading to psoriatic lesions is triggered by T lymphocytes within the epidermal compartment[15].

In summary it seems very likely that proliferation, differentiation and inflammation are integrated processes. Any modulation of one of the processes will interfere in regulation of the others.

5. Future prospectives

The general direction of the lines of investigation presented in this thesis will be maintained. From a theoretical point of view the molecular mechanisms underlying the recruitment of G_0 cells in human epidermis will be explored further as a concept linking the various biochemical and cell biological aspects of the work going on in our department. From a practical aspect, approaches which will receive greater emphasis include (a) use of the new monoclonal antibodies which are becoming available in order to analyze further the heterogeneity of the epidermal cell population, (b) exploration of the possibilities to use hybridocytochemistry directed towards the detection of mRNA, and (c) development and expansion of flow cytometric techniques for studies at the molecular level (calcium, pH, enzymology).

6. References

1. Bauman JGJ, Bentvelzen P Flow cytometric detection of ribosomal RNA in suspended cells by fluorescent in situ hybridization Cytometry 9 517-524, 1988

2 Weinstein GD, McCullough JL Cytokinetics in diseases of epidermal hyperplasia Annu Rev Med 24 345-352, 1973

3. Gelfant S On the existence of non-cycling germinative cells in human epidermis *in vivo* and cell cycle aspects of psoriasis Cell Tissue Kinet 15 393 397, 1982

4 Gelfant S, Dreinko B, Darzynkiewicz Z, Eisenger M, Camplejohn RS Cycling and non-cycling human germinative epidermal cells, the continuous ^{3}H-thymidine labelling method in vivo and flow cytometric studies in Psoriasis cell proliferation Eds Wright NA, Camplejohn RS Edinburgh, Churchill Livingstone, 1983, pp 209 217

5 Boezeman JBM, Bauer FW, de Grood RM Flow cytometric analysis of the recruitment of G_0 cells in human epidermis *in vivo* following tape stripping Cell Tissue Kinet 20 99-107, 1987

6 Wille Jr JJ, Pittelkow MR, Shipley GD, Scott RE Integrated control of growth and differentiation of normal human prokeratinocytes cultured in serum free medium clonal analyses, growth kinetics, and cell cycle studies J Cell Physiol 121 31-44, 1984

7. Albers KM, Setzer RW, Taichman LB Heterogeneity in the replicating population of cultured human epidermal keratinocytes Differentiation 31 134-140, 1986

8 Kanitakis J, Ramirez-Bosca A, Reano A, Viac J, Roche P, Thivolet J Filaggrin expression in normal and pathological skin - a marker of keratinocyte differentiation Virch Arch A Pathol Anat Histopathol 412 375-382, 1988

9. Dover R, Watt FM Measurement of the rate of epidermal terminal differentiation. expression of involucrin by S-phase keratinocytes in culture and in psoriatic plaques J Invest Dermatol 89 349-352, 1987

10. Wille JJ Jr, Pittelkow MR, Shipley GD, Scott RE: Integrated control of growth and differentiation of normal human prokeratinocytes cultured in serum-free medium: Clonal analyses, growth kinetics, and cell cycle studies. J Cell Physiol 121:31-44, 1984

11. Read J, Watt FM: A model for in vitro studies of epidermal homeostasis: Proliferation and involucrin synthesis by cultured human keratinocytes during recovery after stripping off the suprabasal layers. J Invest Dermatol 90:739-743, 1988

12. De Mare S, van Erp PEJ, Ramaekers FCS, van de Kerkhof PCM: Flow cytometric quantification of human epidermal cells expressing keratin 16 in vivo after standardized trauma. Arch Dermatol Res 282:126-130, 1990

13. Hancock GE, Kaplan G, Cohn ZA: Keratinocyte growth regulation by the products of immune cells. J Exp Med 168:1395-1402, 1988

14. Van de Kerkhof: Common pathways for cutaneous inflammation and epidermal proliferation. Thesis, Faculty of Sciences, Nijmegen, 1990

15. Valdimarsson H, Baker BS, Jónsdóttir I, Fry L: Psoriasis: a disease of abnormal keratinocyte proliferation induced by Y lymphocytes. Immunol Today 7:256-259, 1986

CHAPTER IX. SUMMARY, SAMENVATTING

CHAPTER IX. SUMMARY, SAMENVATTING

1. Summary 153
2. Samenvatting 157

1. Summary

Chapter I outlines the aim of the studies presented in this thesis. The aim was to develop novel cytometric techniques to study epidermal growth *in vivo* as well as *in vitro*. Different models for investigation of epidermal growth have been used to test the techniques and to obtain better insight into the processes of epidermal cell division and differentiation. Furthermore, in chapter I an introduction is given to epidermal cell kinetics, keratinocyte differentiation and presently available models for epidermal growth. Finally, cytometric techniques and methodology are introduced to the readers.

Chapter II deals with intermediate filament-type proteins and other differentiation-related proteins as compartment-specific markers in the epidermis. Antibodies have been used to detect these proteins. Immunohistochemical techniques are described to visualize antibody binding and a flow cytometric technique for simultaneous measurement of monoclonal antibody binding and relative DNA content was developed to perform quantitative analysis. It is shown that multiparameter flow cytometry indeed allows quantitative population analysis, that could lead to a better understanding of the complex mechanisms of epidermal growth control under normal and pathologic conditions.

In chapter III an immunocytochemical procedure is described for simultaneous quantification of bromodeoxyuridine (BrdUrd) or iododeoxyuridine (IdUrd) incorporated into cellular DNA and total DNA content in individual cells in suspension. Improvement of existing methods was achieved by combining acid denaturation and proteolytic enzyme digestion. Although experimental conditions were tested on cultured keratinocytes, this method appeared also applicable to bone marrow cells and cells obtained from solid tissues. In order to visualize BrdUrd or IdUrd incorporation into cellular DNA, immunohistochemical techniques were applied to human skin and cultured cells.

Chapter IV describes immunohistochemical and flow cytometrical methods for the detection of a proliferation-associated nuclear antigen recognized by the antibody Ki-67 in human keratinocytes. Using an immunoperoxidase technique on cryostat sections, the antigen was detectable in human epidermis and in cultured keratinocytes. The technique is reproducible, although detection in normal epidermis is often hampered by interference of cytoplasmic staining. An existing flow cytometric method was optimized for precise quantification of Ki-67-positive cells in ethanol-fixed cell suspensions derived from keratinocyte cultures. We were unable to detect Ki-67 antigen in single cell suspensions derived from human skin due to interfering cytoplasmic staining.

Chapter V describes a method to measure intracellular pH in cultured human keratinocytes using flow cytometry. Keratinocytes have the disadvantage that the population is heterogeneous in size. In order to compensate for this cell-to-cell variability in factors such as dye uptake, dyes were selected which change colour with pH. The ratio of fluorescence intensities at two wavelengths was recorded and used as a measure of intracellular pH. It is shown that the novel pH-sensitive dye carboxy-seminaphthorhodafluor-1 (SNARF-1) can be used for flow cytometric pH-measurements. SNARF-1 had a practical and stable excitation wavelength of 488 nm rather than UV, it offered the possibility of ratiometric measurements on the basis of a real emission shift, and had superior resolution for the pH range we are interested in. With SNARF-1 we found that keratinocytes cultured under low serum conditions (0.2%) contain a higher proportion of cells with relative low intracellular pH compared to high serum cultures (6%). Furthermore pH changes were followed by changes in relative DNA content. These findings suggest that intracellular pH can be an early functional proliferation marker for human keratinocytes.

In chapter VI techniques described in previous chapters have been applied to normal and psoriatic epidermis in order to study epidermal growth and differentiation. Additionally, a sequential double immunoenzymatic staining procedure was developed using the monoclonal antibodies anti-BrdUrd and Ki-67 in order to determine whether hyperproliferative skin disorders, such as psoriasis, are characterized by an increased growth fraction rather than a

much shorter cell cycle time of all germinative cells. The ratio of BrdUrd-positive cells over Ki-67-positive cells was not changed in hyperproliferative epidermis compared to normal epidermis. Combining this result with S-phase duration (T_S) calculated from flow cytometric IdUrd/DNA analysis after *in vivo* IdUrd labelling, these findings suggest an increased growth fraction in hyperproliferative epidermis.

Chapter VII deals with cytometric methods described in previous chapters which have been applied to epidermal growth and differentiation *in vitro*. Both processes were studied in exponentially growing and confluent cultures of human keratinocytes. Furthermore, an extended in vitro model was evaluated in which trypsinized keratinocytes derived from confluent cultures were maintained in suspension by continuous rotation. It is shown that with a combination of immunohistochemical and novel flow cytometric techniques a detailed description of epidermal growth and differentiation can be obtained. Under suspension maintenance conditions cells were growth arrested and at the same time changed towards a differentiated phenotype, resembling in many ways normal differentiated cells *in vivo*.

Finally, in chapter VIII findings of the previous chapters were brought together. From a methodological point of view it is concluded that one should not aim for the ultimate technique to measure either proliferation or differentiation, but one should use a panel of phenotypic and functional markers to evaluate these processes. Having used this strategy, we conclude that results presented in this thesis show that differences in tissue turnover and tissue thickness are probably caused by changes in the growth fraction, rather than differences in cell cycle time. Furthermore, it is shown that keratin 16 expression in suprabasal cells *in vivo* and in cultured keratinocytes is not a characteristic of hyperproliferation per se, but instead is reflecting a condition in which synchronization of differentiation steps is lost. It is proposed that keratin 16 expression in psoriasis is also reflecting asynchronous differentiation and is caused by the overproduction of basal keratinocytes. The overproduction itself is a result of a defect in the $G_0 \rightarrow G_1$ recruitment mechanism.

2. Samenvatting

In hoofdstuk I wordt de doelstelling van het onderzoek uiteengezet. Het doel van de verschillende studies was in de eerste plaats de ontwikkeling van cytometrische technieken, waarmee *in vivo* en *in vitro* epidermale groei kan worden bestudeerd. Om de bruikbaarheid van de nieuwe technieken te testen en om een beter inzicht te krijgen in epidermale celdeling en differentiatie, werden verschillende modellen voor epidermale groei gebruikt. Verder worden in hoofdstuk I begrippen als epidermale cel kinetiek en differentiatie van keratinocyten geïntroduceerd en worden op dit moment gebruikte modellen voor epidermale groei besproken. Tenslotte wordt een inleiding gegeven met betrekking tot cytometrische technieken en methodologie.

Intermediaire filamenteiwitten en andere aan differentiatie gerelateerde eiwitten zijn het onderwerp van hoofdstuk II. Hierbij wordt in het bijzonder ingegaan op hun rol als compartiment-specifieke merkers in de epidermis. Om de eiwitten te detecteren werden antilichamen gebruikt en immunohistochemische technieken worden beschreven om de binding van deze antilichamen te visualiseren. Verder werd een flowcytometrische techniek ontwikkeld om simultaan binding van antilichamen en relatieve DNA hoeveelheid te meten, waardoor kwantitatieve analyse mogelijk werd. In hoofdstuk II wordt aangetoond dat multiparameter flowcytometrie inderdaad gebruikt kan worden voor kwantitatieve populatie-analyse en dat die metingen kunnen leiden tot een beter begrip van de complexe mechanismen, die ten grondslag liggen aan epidermale groeicontrole onder normale en pathologische condities.

In hoofdstuk III wordt een nieuwe immunocytochemische procedure beschreven, die de mogelijkheid biedt om simultaan in het DNA ingebouwd bromodeoxyuridine (BrdUrd) of jododeoxyuridine (IdUrd) en de totale DNA hoeveelheid te meten in celsuspensies. Omdat bestaande technieken niet of nauwelijks bruikbaar bleken voor keratinocyten werd gezocht naar een alternatieve techniek. Uiteindelijk werd een verbetering gerealiseerd die zure denaturatie combineerde met enzymatische proteolyse. Hoewel de experimentele condities werden uitgezocht met gekweekte keratinocyten, bleek de methode ook bruikbaar voor cellen afkomstig uit het beenmerg en solide

weefsels. Om BrdUrd of IdUrd inbouw in het DNA zichtbaar te maken werden immunohistochemische technieken toegepast.

Hoofdstuk IV beschrijft immunohistochemische en flowcytometrische methoden om een proliferatie-geassocieerd kernantigeen aan te tonen in humane keratinocyten door middel van het antilichaam Ki-67. Met behulp van een immunoperoxidase techniek was het antigeen aan te kleuren in humane epidermis en in gekweekte keratinocyten. De techniek bleek reproduceerbaar, hoewel detectie in normale epidermis vaak werd bemoeilijkt door interferentie van cytoplasmaire kleuring. Een eerder beschreven flowcytometrische techniek werd geoptimaliseerd voor de precieze kwantificering van Ki-67-positieve cellen in ethanol-gefixeerde celsuspensies afkomstig van keratinocytenkweken. Het bleek niet mogelijk om Ki-67 antigeen aan te tonen in celsuspensies afkomstig van humane huid, opnieuw door de interfererende cytoplasmaire kleuring.

Hoofdstuk V beschrijft een methode om flowcytometrisch de intracellulaire pH (pH_i) te meten in gekweekte keratinocyten. Keratinocyten hebben het grote nadeel dat de populatie wat betreft celgrootte erg heterogeen is. Om te kunnen compenseren voor deze cel tot cel variabiliteit werden pH-gevoelige fluorochromen gekozen die van kleur veranderen als de pH verandert. De ratio van fluorescentie signalen gemeten bij twee excitatie golflengten werd gebruikt als een maat voor de pH_i. Aangetoond wordt dat de recent geïntroduceerde pH-gevoelige kleurstof carboxy-seminaphthorhodafluor-1 (SNARF-1) gebruikt kan worden voor flowcytometrische pH_i-metingen. SNARF-1 bleek een praktische en stabiele excitatiegolflengte te bezitten van 488 nm. Het emissiespectrum vertoonde een reële pH-afhankelijke verschuiving en de pH resolutie was superieur in het gebied waarin wij zijn geïnteresseerd. Met behulp van SNARF-1 waren we in staat om aan te tonen dat in een keratinocytenpopulatie, die gekweekt werd met een lage serum concentratie (0.2%), meer cellen aanwezig waren met een relatief lage pH_i dan in een populatie gekweekt met een standaard serum concentratie (6%). Verder werden deze pH_i veranderingen gevolgd door veranderingen in relatieve DNA hoeveelheid. Deze bevindingen suggereren dat pH_i gebruikt kan worden als een vroege proliferatie merker voor epidermale cellen.

In hoofdstuk VI worden technieken, die werden beschreven in de hoofd-

stukken II-V, toegepast op normale en psoriatische epidermis ter bestudering van epidermale groei en differentiatie. Daarnaast werd een sequentiële immuno-enzymatische dubbelkleuring ontwikkeld, gebruik makend van de monoclonale antilichamen anti-BrdUrd en Ki-67, om aan te tonen dat hyperproliferatieve huidaandoeningen zoals psoriasis worden gekenmerkt door een toegenomen groeifraktie in plaats van een verkorte cel cyclus tijd van alle germinatieve cellen. Vergeleken met normale epidermis bleek de ratio van anti-BrdUrd-positieve en Ki-67-positieve cellen niet veranderd in hyperproliferatieve epidermis. Dit resultaat, gecombineerd met de S-fase duur, berekend uit de flowcytometrische IdUrd/DNA analyses na *in vivo* IdUrd labeling, suggereert een toegenomen groeifraktie in hyperproliferatieve epidermis.

Hoofdstuk VII behandelt cytometrische methoden, beschreven in eerdere hoofdstukken, die worden toegepast om *in vitro* groei en differentiatie te bestuderen. Groei en differentiatie werden onderzocht in exponentieel groeiende en confluente keratinocytenkweken. Verder werd een uitbreiding van de kweekcondities getest, waarbij getrypsineerde keratinocyten, afkomstig van confluente celkweken, roterend in suspensie werden aangehouden. Met behulp van een combinatie van immunohistochemische en flowcytometrische technieken bleken wij in staat om een gedetailleerde beschrijving te geven van groei en differentiatie onder de verschillende kweekcondities. Wanneer cellen in suspensie werden aangehouden verloren ze hun capaciteit om te delen terwijl tegelijkertijd een meer gedifferentieerd fenotype geëxprimeerd werd wat in veel opzichten overeenkomst vertoonde met normaal gedifferentieerde cellen *in vivo*.

In hoofdstuk VIII tenslotte worden de belangrijkste bevindingen uit hoofdstuk II-VII nogmaals bijeen gebracht. Methodologisch bekeken moet geconcludeerd worden dat het gebruik van een panel van fenotypische en functionele merkers om proliferatie en differentiatie te meten valt te prefereren boven het zoeken naar de volmaakte techniek en merker die of proliferatie of differentiatie kan meten. Gebruik makend van deze strategie kan vervolgens geconcludeerd worden dat de resultaten met betrekking tot groei en differentiatie van keratinocyten, die gepresenteerd worden in dit proefschrift, aantonen dat verschillen in weefsel turnover en dikte naar alle waarschijnlijkheid worden veroorzaakt door veranderingen in de groeifraktie in plaats van veranderingen

in celcyclus duur. Verder wordt aangetoond dat expressie van keratine 16 in suprabasale cellen *in vivo* en in gekweekte keratinocyten geen kenmerk is van hyperproliferatie per se, maar daarentegen een toestand reflecteert waarin de synchronisatie van differentiatie stappen verloren is gegaan. Deze bevindingen hebben geleid tot de hypothese dat expressie van keratine 16 in psoriasis het gevolg is van het niet synchroon verlopen van differentiatiestappen en dat deze asynchrone differentiatie veroorzaakt wordt door de massale overproduktie van basale keratinocyten. De overproduktie zelf is op haar beurt het resultaat van een defect in het $G_0 \rightarrow G_1$ recruteringsmechanisme.

Gommans JM, Bergers M, Erp PEJ van, Hurk JJMA van den, Mier PD, Roelfzema H.
Studies on the plasma membrane of normal and psoriatic keratinocytes. I. Preparation of material and morphological characterization.
Br J Dermatol 1979; 101:407-412.

Gommans JM, Bergers M, Erp PEJ van, Hurk JJMA van den, Kerkhof PCM van de, Mier PD, Roelfzema H.
Studies on the plasma membrane of normal and psoriatic keratinocytes. 2. Cyclic AMP and its response to hormonal stimulation.
Br J Dermatol 1979; 101:413-419.

Geerdink JPM, Bergers M, Erp PEJ van, Gommans JM, Mier PD, Roelfzema H.
Cyclic AMP is decreased in mononuclear leukocytes from psoriasis patients.
Br J Dermatol 1980; 103: 107-108.

Roelfzema H, Bergers M, Erp PEJ van, Gommans JM, Mier PD.
Studies on the plasma membrane of normal and psoriatic keratinocytes. 3. Uptake of labelled sugars and their incorporation into glycoconjugates.
Br J Dermatol 1981; 104: 635-640.

Roelfzema H, Bergers M, Erp PEJ van, Gommans JM, Mier PD.
Studies on the plasma membrane of normal and psoriatic keratinocytes. 4. Characterization of glycoconjugates.
Br J Dermatol 1981; 105:509-516.

Gommans JM, Hurk JJMA van den, Bergers M, Erp PEJ van, Mier PD, Roelfzema H.
Studies on the plasma membrane of normal and psoriatic keratinocytes. 5. Lectin binding.
Br J Dermatol 1982; 106:317-322.

Mier PD, Rennes H van, Erp PEJ van, Roelfzema H.
Cutaneous sialidase.
J Invest Dermatol 1982; 78:267-269.

Mier PD, Rennes H van, Erp PEJ van, Roelfzema H.
Alpha-l-fucosidase in normal and psoriatic epidermis.
Br J Dermatol 1982; 106:367-368.

Feyen JHM, Erp PEJ van, Mier PD.
Rapid technique for the radiometric assay of phospholipase A_2
J Chromatogr 1983; 259:338-340.

Kerkhof PCM van de, Erp PEJ van.
Calmodulin levels are grossly elevated in the psoriatic lesion.
Br J Dermatol 1983; 108:217-218.

Roelfzema H, Erp PEJ van.
Studies on the plasma membrane of normal and psoriatic keratinocytes. 6. Cell surface and shed glycoproteins.
J Invest Dermatol 1983; 80:24-26.

Roelfzema H, Erp PEJ van.
Glycoprotein composition of psoriatic epidermis in relation to growth control.
J Invest Dermatol 1983; 80:20-23.

Erp PEJ van, Bergers M, Grood RM de, Koedam J, Rijntjes JM.
Fucosyl glycopeptide profiles of keratinocytes from various epithelial tissues of the rabbit in relation to differentiation in vivo and in vitro.
J Invest Dermatol 1984; 83:359-362.

Geerding JPM, Mulder PHM, Franzen MR, Gommans JM, Rennes H, Erp PEJ van, Bergers M, Mier PD.
Monocyte function is normal in quiescent psoriasis.
J Invest Dermatol 1984; 82:122-125.

Verhagen A, Bergers M, Erp PEJ van, Gommans JM, Kerkhof PCM van de, Mier PD.
Confirmation of raised phospholipase A2 activity in the uninvolved skin of psoriasis.
Br J Dermatol 1984; 110:731-732.

Gommans JM, Erp PEJ van, Forster S, Boezeman J, Mier PD.
Isolation and preliminary biochemical characterization of the human epidermal Langerhans cell.
J Invest Dermatol 1985; 85:191-193.

Kerkhof PCM van de, Erp PEJ van.
Epidermal calmodulin and skin disease.
Int J Dermatol 1985; 24:507-508.

Roelfzema H, Erp PEJ van, Mier PD.
Glycoprotein metabolism in skin cancer: synthesis, pool size, and partial characterization of glycoproteins in the rat basosquamous cell carcinoma.
J Invest Dermatol 1985; 85:235-238.

Erp PEJ van, Mier PD.
Molecular Biology.
In: Textbook of Psoriasis. Eds. Mier PD, Kerkhof PCM van de.
Churchill Livingstone, Edinburgh 1986; pp 125-149.

Meeren HLJ van der, Erp PEJ van.
Life-threatening contact urticaria from glove powder.
Contact Dermatitis 1986; 14:190-191.

Erp PEJ van, Kerkhof PCM van de.
Calmodulin levels in psoriasis and other skin disorders.
Arch Dermatol Res 1987; 279:151-153.

Bokhoven M van, Mare S de, Czarnetzki BM, Erp PEJ van, Boezeman JBM, Kerkhof PCM van de.
Active vitamin D3 does not suppress recruitment of G_0 cells following standardized injury.
Br J Dermatol 1988; 119:737-742.

Erp PEJ van, Kerkhof PCM van de.
Epidermal calmodulin levels and reference variables.
J Invest Dermatol 1988; 90:536-537.

Erp PEJ van, Brons PPT, Boezeman JBM, Jongh GJ de, Bauer FW.
A rapid flow cytometric method for bivariate bromodeoxyuridine/DNA analysis using simultaneous proteolytic enzyme digestion and acid denaturation.
Cytometry 1988; 9:627-630.

Rappard JHA van, Bauer FW, Grubben MJAL, Erp PEJ van, Borghouts JMHM.
Epidermopoiesis in controlled tissue-expansion.
Chapter 4, Thesis, Groningen 2 maart 1988.

Beurskens T, Chang A, Erp PEJ van, Kerkhof PCM van de.
Epidermal proliferation and accumulation of polymorphonuclear leukocytes in the psoriatic lesion.
Dermatologica 1989; 178:67-72.

Erp PEJ van, Mare S de, Rijzewijk JJ, Kerkhof PCM van de, Bauer FW.
A sequential double immunoenzymatic staining procedure to obtain cell kinetic information in normal and hyperproliferative epidermis.
Histochem J 1989; 21:343-347.

Erp PEJ van, Rijzewijk JJ, Boezeman JBM, Leenders J, Mare S de, Kerkhof PCM van de, Ramaekers FCS, Bauer FW.
Flow cytometric analysis of epidermal subpopulations from normal and psoriatic skin using monoclonal antibodies against intermediate filament.
Am J Pathol 1989; 135;865:870.

Mare S de, Erp PEJ van, Kerkhof PCM van de.
Epidermal hyperproliferation assessed by the monoclonal antibody Ks8.12 on frozen sections.
J Invest Dermatol 1989; 92:130-131.

Mare S de, Hartog G den, Calis N, Erp PEJ van, Kerkhof PCM van de
A double blind study on the relevance of salicylic acid in dithranol creams, monitored by clinical assessment and quantification of hyperproliferation markers
Skin Pharmacol 1989, 1 259 264

Rijzewijk JJ, Erp PEJ van, Bauer FW
Two binding sites for Ki67 related to quiescent and cycling cells in human epidermis
Acta Derm Venereol (Stockh) 1989, 69.512-515

Rijzewijk JJ, Erp PEJ van, Boezeman JBM, Jongh GJ de, Bauer FW
The development of the bromodeoxyuridine technique for kinetic studies in human epidermis
Epithelia 1989, 1 323-333

Steijlen PM, Hamm H, Erp PEJ van, Johnson JP, Ruiter DJ, Bröcker EB
Immunohistologic evidence for the malignant potential of congenital melanocytic nevi
J Invest Dermatol 1989, 92 366-370

Brons PPT, Erp PEJ van, Pennings AHM, Boezeman JBM
Cell kinetics in acute myeloid leukemia and other hematological malignancies studied with in vivo iododeoxyuridine administration and flow cytometry
In New Trends in Acute Leukemia PAOG University of Nijmegen, Nijmegen 1990, pp 49-58

Mare S de, Erp PEJ van, Ramaekers FCS, Kerkhof PCM van de
Flow cytometric quantification of human epidermal cells expressing keratin 16 in vivo after standardized trauma
Arch Dermatol Res 1990, 282 126-130

Mare S de, Jong EMGJ de, Erp PEJ van, Kerkhof PCM van de
Markers for proliferation and keratinization in the margin of the active psoriatic lesion
Brit J Dermatol 1990, 122 469-475

Mare S de, Erp PEJ van, Jong EMGJ de, Kerkhof PCM van de
DNA content and K_s8 12 binding of the psoriatic lesion during treatment with the vitamin D3 analogue MC903 and betamethasone
Br J Dermatol 1990, 123 291 295

Erp PEJ van, Jansen MJJM, Jongh GJ de, Boezeman JBM, Schalkwijk J
Flow cytometric measurement of intracellular pH in cultured human keratinocytes using carboxy-SNARF 1
In Advances in analytical cellular pathology Eds Burger G, Oberholzer M, Vooijs GP
Excerpta Medica 1991, pp 131 132

Erp PEJ van, Jansen MJJM, Jongh GJ de, Boezeman JBM, Schalkwijk J
Ratiometric measurement of intracellular pH in cultured human keratinocytes using carboxy-SNARF-1 and flow cytometry
Cytometry 1991, 12 127 132

Jong EMGJ de, Erp PEJ van, Vlijmen IMJJ van, Kerkhof PCM van de.
Cutaneous inflammation and proliferation induced by leukotriene B_4.
In: Pharmacology of the Skin (Karger); 4th Volume 1991; in press.

Jong EMGJ de, Erp PEJ van, Vlijmen IMMJ van, Kerkhof PCM van de.
The interrelation between inflammation and epidermal proliferation in normal skin following epicutaneous application of leukotriene B_4: an immunohistochemical study.
Clin Exp Dermatol 1991; in press

Jong EMGJ de, Vlijmen IMMJ van, Erp PEJ van, Ramaekers FCS, Troyanovski SM, Kerkhof PCM van de.
Keratin 17: a useful marker in antipsoriatic therapies.
Arch Dermatol Res 1991; in press.

Jong EMGJ de, Erp PEJ van, Ruiter DJ, Kerkhof PCM van de.
Immunopathology of proliferation and differentiation in discoid lupus erythematosus.
submitted.

Steen PHM van der, Boezeman JBM, Erp PEJ van, Vlijmen IMJJ van, Kerkhof PCM van de.
Epidermal proliferation and keratinization following standardized elicitation with diphenyl-cyclopropenone.
submitted.

Vlijmen IMMJ van, Erp PEJ van.
Use of microwave irradiation for rapid and enhanced immunohistochemical staining: application to skin antigens.
submitted.

DANKWOORD

Op deze plaats wil ik een ieder bedanken die meegeholpen heeft aan het tot stand komen van dit proefschrift. Met name wil ik mijn collega's Gijs de Jongh en Ivonne van Vlijmen danken voor hun praktische en mentale ondersteuning. Zonder de collegialiteit van Ivonne zou de promotiedatum zeker later zijn geweest en zonder Gijs en zijn onnavolgbare manier van celkweken zou de helft van het in dit proefschrift beschreven onderzoek niet uitgevoerd zijn.

Met betrekking tot de andere helft van het onderzoek gaat mijn bijzondere dank uit naar de vele vrijwilligers, met name familie en kennissen, die een stukje (huid) van hun zelf gaven voor de wetenschap en mijn proefschrift.

Zonder uitzondering wil ik de stafleden van de afdeling Dermatologie danken dat ze mij de ruimte hebben geboden om dit promotieonderzoek uit te voeren. Zonder de anderen daarmee tekort te doen, wil ik toch een paar mensen met name noemen. Hoewel hij het zich waarschijnlijk niet gerealiseerd heeft, was het Henk Roelfzema die, tijdens zijn postdoc-periode 1978-1982 in "de kelder van Neurologie", met zijn pure wetenschapsfilosofie mij op de weg naar dit proefschrift heeft gezet. Het waren Franz Bauer en Jan Boezeman die enkele jaren later aan de basis stonden van wat nu gerealiseerd is in dit proefschrift. Tenslotte was het Joost Schalkwijk die op zijn eigen nuchtere wijze zorg droeg voor de "finishing touch".

Waltraud Verbeeck wil ik niet alleen danken voor de (tekst)verwerking van manuscripten, abstracts en posters, maar ook voor de plezierige periode dat we de werkplek en PC in het "tussenhok" deelden.

De (ex-)studenten Joris Rijzewijk, John Leenders, Hennie Groenendal, Saskia de Mare, Resy Ramaekers, Monique Jansen en Marc Berndsen hebben een significante bijdrage geleverd aan dit proefschrift.

Veel dank (en tijd) ben ik ook verschuldigd aan Ans en Anneke. Zonder hun aanhoudende steun waren de inspanningen van de afgelopen jaren onmogelijk geweest.

Piet van Erp werd geboren op 24 maart 1956 in het brabantse Geffen. Na het behalen van het eindexamen HAVO in 1973 aan het Titus Brandsma-lyceum te Oss begon hij een hogere beroepsopleiding aan de Hogere en Middelbare Laboratorium School in diezelfde stad. In het kader van de specialisatie HBO-B biochemie werd een stageperiode doorlopen bij de N.V. Philips Gloeilampenfabrieken te Eindhoven. Op de afdeling plantenfysiologie (dr. G. Engelsma) werd onderzoek verricht naar de relatie tussen fenolmetabolisme en bloei-inductie in planten. Nadat in 1976 het diploma HBO-B biochemie werd behaald, werd de militaire dienstplicht vervuld.

Sinds 2 april 1978 is de auteur van dit proefschrift verbonden aan de afdeling Dermatologie van het Academisch Ziekenhuis Nijmegen (waarnemend hoofd: dr. P.C.M. van de Kerkhof). Van 2 april 1978 tot 1 oktober 1980 was er een aanstelling in tijdelijk dienstverband op basis van een KWF-project, getiteld "Plasma membrane and epidermal proliferation". Vanaf 1 oktober 1980 werd het tijdelijk dienstverband omgezet in een aanstelling voor onbepaalde tijd. Vanaf 1 mei 1986 is hij betrokken bij onderzoek naar groei, groeiregulatie en celkinetiek van de huid waarbij, naast immunohistochemische technieken, vooral gebruik wordt gemaakt van flowcytometrie. Een deel van dit werk is verwerkt in dit proefschrift.

In 1979 is hij getrouwd met Ans Jans en hij is de trotse vader van de zesjarige Anneke.